Journal of Visualized Experiments

Preparation of expanded chitin foams and their use in the removal of aqueous copper

--Manuscript Draft--

Article Type:	Methods Article - JoVE Produced Video
Manuscript Number:	JoVE62301R1
Full Title:	Preparation of expanded chitin foams and their use in the removal of aqueous copper
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Additional Information:	
Question	**Response**
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TITLE:

Preparation of Expanded Chitin Foams and their Use in the Removal of Aqueous Copper

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KEYWORDS:

adsorption, copper, chitin, biopolymer, expanded polymer, polymer foam

SUMMARY:

This study describes a method to expand chitin into a foam by chemical techniques that require no specialized equipment.

ABSTRACT:

Chitin is an underexploited, naturally abundant, mechanically robust, and chemically resistant biopolymer. These qualities are desirable in an adsorbent, but chitin lacks the necessary specific surface area, and its modification involves specialized techniques and equipment. Herein is described a novel chemical procedure for expanding chitin flakes, derived from shrimp shell waste, into foams with higher surface area. The process relies on the evolution of H_2 gas from the reaction of water with NaH trapped in a chitin gel. The preparation method requires no specialized equipment. Powder X-ray diffraction and N_2-physisorption indicate that the crystallite size decreases from 6.6 nm to 4.4 nm and the specific surface area increases from 12.6 ± 2.1 m^2/g to 73.9 ± 0.2 m^2/g. However, infrared spectroscopy and thermogravimetric analysis indicate that the process does not change the chemical identity of the chitin. The specific Cu adsorption capacity of the expanded chitin increases in proportion to specific surface area from 13.8 ± 2.9 mg/g to 73.1 ± 2.0 mg/g. However, the Cu adsorption capacity as a surface density remains

relatively constant at an average of 10.1 ± 0.8 atom/nm^2, which again suggests no change in the chemical identity of the chitin. This method offers the means to transform chitin into a higher surface area material without sacrificing its desirable properties. Although the chitin foam is described here as an adsorbent, it can be envisioned as a catalyst support, thermal insulator, and structural material.

INTRODUCTION:

Chitin is a mechanically robust and chemically inert biopolymer, second only to cellulose in natural abundance[1]. It is the major component in the exoskeleton of arthropods and in the cell walls of fungi and yeast[2]. Chitin is similar to cellulose, but with one hydroxyl group of each monomer replaced with an acetyl amine group (**Figure 1A,B**). This difference increases the strength of hydrogen bonding between adjacent polymer chains and gives chitin its characteristic structural resilience and chemical inertness[2,3]. Due to its properties and abundance, chitin has attracted significant industrial and academic interest. It has been studied as a scaffold for tissue growth[4–6], as a component in composite materials[7–11], and as a support for adsorbents and catalysts[11–14]. Its chemical stability, in particular, makes chitin attractive for adsorption applications that involve conditions inhospitable to common adsorbents[14]. In addition, the abundance of amine groups make chitin an effective adsorbent for metal ions[15]. However, the protonation of the amine groups under acidic conditions reduces the metal adsorption capacity of chitin[16]. A successful strategy is to introduce adsorption sites more resistant to protonation[17,18]. Instead, herein is described a simple method to increase the specific surface area and, therefore, the number of adsorption sites in chitin.

[Place **Figure 1** here]

In spite of its many potential uses, chitin is underutilized. Chitin processing is challenging due to its low solubility in most solvents. A key limitation to its use in catalysis and adsorption is its low specific surface area. While typical carbon and metal oxide supports have specific surface areas in the order 10^2–10^3 m^2/g, commercial chitin flakes have surface areas in the order of 10 m^2/g[19–21]. Methods to expand chitin into foams exist, but they invariably rely on high temperature and pressure, strong acids and bases, or specialized equipment that represent a significant entry barrier[5,21–25]. In addition, these methods tend to deacetylate chitin to form chitosan (**Figure 1C**)—a more soluble and reactive biopolymer[5, 25,26].

Herein, a method is described to expand chitin into solid foams, increase its specific surface area and adsorption capacity, and maintain its chemical integrity. The method relies on the rapid evolution of gas from within a chitin gel and requires no specialized equipment. The increased adsorption capacity of the expanded chitin is demonstrated with aqueous Cu^{2+}—a common contaminant in the local groundwater[26].

[Place **Table 1** here]

PROTOCOL:

1. Preparation of expanded chitin

1.1. Prepare a 250 mL solution of 5 wt% LiCl in dimethylacetamide (DMAc)

CAUTION: The solvent DMAc is a combustible irritant that may damage fertility and cause birth defects. Handle DMAc in a fume hood using chemical resistant gloves and goggles to avoid contact with skin and eyes.

1.1.1. Add 15 g of LiCl and 285 g (268 mL) of DMAc into a 500 mL Erlenmeyer flask with, then place a 50 mm Polytetrafluoroethylene (PTFE)-lined magnetic stir bar.

1.1.2. Cap the flask with a rubber septum and place it on a heating stir plate. Place a temperature probe through the septum into the mixture. Stir the mixture at 400 rpm and 80 °C until all LiCl is dissolved (~ 4 h)

1.2. Dissolve 1.0 g of oven-dried chitin flakes in the LiCl/DMAc solution to form a sol-gel

1.2.1. Dry at least 1.2 g of chitin flakes in an oven at 80°C for 24 h.

1.2.2. Add 1.0 g of oven-dried chitin flakes and 250 mL of 5 wt% LiCl/DMAc solution into a 500 mL round bottom flask. Place a 50 mm PTFE-lined magnetic stir bar.

1.2.3. Cap the flask with a rubber septum and place it on a stirring heat block. Pierce the septum with a needle and leave it to allow the flask to vent. Heat the block to 80 °C and stir the mixture at 400 rpm until all the chitin is dissolved (24–48 h).

1.2.4. Allow the resultant chitin sol-gel to cool down to room temperature slowly while continuing to stir (~ 1 h).

1.2.5. Once at room temperature, place the flask containing the chitin sol-gel in an ice bath and continue stirring until its temperature stabilizes (~ 20 min).

1.3. Prepare a 100 mL slurry of NaH in DMAc.

CAUTION: NaH in contact with water releases flammable gases which may ignite spontaneously. To limit contact with moist air, NaH is stored in mineral oil which must be washed off before use. Handle with caution in a fume hood using chemical resistant gloves and goggles.

1.3.1. Remove approximately 1 g of NaH from its mineral oil storage and wash three times with 10 mL of hexanes.

1.3.2. Add 100 mL of DMAC into a 250 mL Erlenmeyer flask, then add 0.82 g of the washed NaH and place a PTFE-lined magnetic stir bar.

1.3.3. Swirl the mixture to produce a NaH/DMAc slurry.

NOTE: NaH will not completely dissolve.

1.4. Form the chitin gel by adding all the NaH/DMAc slurry to the chitin sol-gel.

1.4.1. Uncap the cooled sol-gel and add all the NaH slurry while stirring vigorously. Replace the cap and continue to stir the mixture at 400 rpm for 72 h or until a gel forms in the flask.

1.5. Form the chitin foam by adding water to the chitin gel.

1.5.1. After the formation of the gel, uncap the flask and add 100 mL of Deionized (DI) water.

NOTE: It is critical to perform this step in a fume hood as the process will evolve H_2 gas.

1.6. Isolate, and wash the chitin foam in water and methanol to remove DMAc and salts.

1.6.1. Remove the expanded chitin foam from the flask and place in a crystallization dish or beaker sufficiently large to hold it and 1000 mL of DI water.

NOTE: The chitin foam will not come out in one piece and may have to be broken up.

1.6.2. Rinse the isolated gel three times with 500 mL of DI water. Soak the gel in 1000 mL of DI water for 24 h, then in 500 mL of methanol for 24 h, and finally in 1000 mL of DI water for 24 h again.

1.6.3. Remove the expanded chitin foam from the water wash and allow to air dry for 24-48 h.

1.7. Dry the washed chitin gel to form a solid foam and then grind to a powder.

1.7.1. Dry the gel in the oven at 85 °C for 48 h under ambient air, or in a lyophilizer at -43 °C and 0.024 mbar for 48 h.

1.7.2. Using a mortar and pestle, grind the dry chitin foam into a fine powder.

[Place **Figure 2** here]

2. Development of the adsorption isotherms

2.1. Prepare 500 mL stock solutions of aq. Cu^{2+} (MW 63.5 g/mol) at concentrations 50 mg/L, 100 mg/L, 200 mg/L, 300 mg/L, 400 mg/L, and 450 mg/L. To do this, add 90 mg, 180 mg, 360 mg, 540 mg, 720 mg, and 810 mg of $Cu(NO_3)_2 \cdot 2.5\ H_2O$ (MW 232.6 g/mol) to six containers, respectively. Add 500 mL of 18 MΩ water, cap the container, and shake to dissolve the solids.

2.2. Add 50 mg of chitin to 100 mL of each stock solution, adjust the pH to 7, and allow to equilibrate for 48 h.

2.2.1. Transfer 100 mL of each stock solution to a 100 mL container so the headspace is minimal. Add 50 mg of ground chitin to each container and then cap them.

2.2.2. Place containers on an orbital shaker and shake at 60 rpm for 30 min. Then take containers off the orbital shaker and adjust the pH to 7 using NH_4HCO_3 or HNO_3.

2.2.3. Replace containers back on the orbital shaker and shake at 60 rpm and at a constant temperature for 48 h. Maintain the laboratory at 18 ± 2 °C throughout.

2.3. Measure the Cu concentration of the initial stock solutions and of those to which chitin was added. Use the colorimetric bicinchoninate method, a colorimeter, and pre-measured reagent packets[27].

2.3.1. Remove containers from the orbital shaker, allow the mixtures to settle for a minimum of 30 min, and then take a 1 mL aliquot with a syringe fitted with a 0.3 µm glass microfiber filter.

2.3.2. Transfer the aliquot to a 250 mL container and dilute to 100 mL with 18 MΩ water.

NOTE: This step is necessary due to the low ceiling of detection of Cu (5 mg/L) by the bicinchoninate method using the colorimeter.

2.3.3. Transfer 10 mL of the diluted sample to a cuvette. Place the cuvette in the colorimeter and zero the instrument.

2.3.4. Add one packet of premeasured Cu reagent (bicinchoninate method) to the diluted sample in the cuvette and wait 45 s for the chelation reaction to complete. Allow the solution to become purple. The intensity of the color formed is proportional to the Cu concentration.

2.3.5. Place the cuvette back in the colorimeter and measure the Cu concentration of the diluted sample. Multiply the concentration of the diluted sample by 100 to obtain that of the original sample.

2.4. Extract the maximum Cu uptake from the adsorption isotherm data.

2.4.1. Calculate the uptake of each sample for each equilibrium Cu concentration using the equation[28]:

$$Uptake\left(\frac{mg_{Cu}}{g_{Chitin}}\right) = \frac{Volume\ (L)}{Mass\ (g_{Chitin})} \cdot \left(Initial\ Conc.\left(\frac{mg_{Cu}}{L}\right) - Equilibrium\ Conc.\left(\frac{mg_{Cu}}{L}\right)\right)$$

2.4.2. Plot the adsorption uptake versus equilibrium concentration of the samples to produce a standard Cu adsorption isotherm.

2.4.3. Plot the ratio of equilibrium concentration to uptake versus the equilibrium concentration to produce the linearized Cu adsorption isotherm.

NOTE: The plot should be linear, and the inverse of the slope represents the maximum Cu uptake.

REPRESENTATIVE RESULTS:

Expanded chitin shows the same morphology regardless of the drying method. **Figure 3** shows images of neat chitin flakes (**Figure 3A1**), oven-dried expanded chitin (**Figure 3B1**), and lyophilized expanded chitin (**Figure 3C3**). While the neat flakes have the appearance of coarse sand, the expanded chitin foam has the appearance of a kernel of popped corn. Scanning electron micrographs show a similar change at smaller scales. While the neat chitin flakes (**Figure 3A2,3A3**) have a compact, dense structure, the oven dried (**Figure 3B2,3B3**) and lyophilized (**Figure 3C2,3C3**) expanded chitin resemble crinkled paper or wrinkled sheets. The samples were sputter coated with gold before imaging with a secondary electron detector, with a 15 kV accelerating voltage, and at a working distance in the range of 29–31 mm.

[Place **Figure 3** here]

These visual and microscopic observations agree with the powder X-ray Diffraction (XRD) and N_2-physisorption analyses of the samples. Diffractograms show a widening of crystalline reflections and an increase in the intensity of the amorphous peak in the expanded foams relative to the neat flakes (**Figure 4**). This observation can be illustrated by comparing the semi-quantitative crystallinity index and the crystallite size estimates of the neat and expanded chitin. The crystallinity index is the normalized difference of crystalline to amorphous diffraction intensities[29]. It is given by the equation:

$$Crystallinity\ Index = 100 \cdot \frac{Crystalline\ Intensity - Amorphous\ Intensity}{Crystalline\ Intensity}$$

For chitin, the crystalline diffraction intensity typically used is that of crystal plane (110) at 19.3° and the amorphous diffraction intensity is that at 16.0°[29]. The crystallinity index drops from 88% in the neat flakes, to 74% in the oven-dried expanded foam, and to 58% in the lyophilized expanded foam (**Table 1**). The crystallite size can be estimated by the Scherrer equation[30]:

$$Crystallite\ Size\ (nm) = \frac{Shape\ factor \cdot X\ Ray\ Wavelength\ (nm)}{Full\ Width\ at\ Half\ Maximum\ (radians) \cdot \cos(Angle\ (radians))}$$

We assume a shape factor of 1 and the instrument used Cu K_α radiation (wavelength = 15.4 nm). Using the diffraction of the (110) plane at 19.3°, the crystallite size drops from 6.6 nm in the neat chitin to 4.4 nm in the expanded chitin (**Table 1**).

[Place **Figure 4** here]

Measurements of specific surface area, obtained from N_2-physisorption isotherms at 77 K using the Brunauer-Emmett-Teller (BET) equation[31], lead to similar observations. For all the materials, the N_2 adsorption isotherms show the uptake volume to increase linearly with partial pressure in the range $p/p_o = 0.05\text{-}0.25$ (**Figure 5A**), as is expected of N_2 multilayer condensation[32]. However, the uptake volume is greatest for the expanded foams. The BET plot (**Figure 5B,5C**), show a positive linear correlation with partial pressure and positive intercept, indicating that the data is within the valid range of the BET equation[33]. As such, the specific surface area of the materials is proportional to the inverse of the sum of the slope and intercept of those lines[31]. While the specific surface area of the neat flakes is 12.6 ± 2.1 m^2/g, that of oven dried foam is 43.1 ± 0.2 m^2/g, and that of the lyophilized foam is 73.9 ± 0.2 m^2/g. The changes in crystallinity index, crystallite size, and specific surface area indicate that the material either (1) forms a more open and porous structure, or (2) is degraded into smaller particles. The micrographs in **Figure 3** suggest the former, but the latter cannot be ruled out without a thorough pore-size distribution analysis.

[Place **Figure 5** here]

In spite of the morphological changes described above, the expansion process does not appear to affect the chemical structure of chitin. The IR spectrum, obtained as attenuated total reflectance (ATR), of all chitin samples remain virtually unchanged regardless of processing (**Figure 6**). Note the similarity of the peaks at 1650 cm^{-1} and 1550 cm^{-1} which correspond to the amide functional group[23].

[Place **Figure 6** here]

The thermal decomposition behavior also indicates minimal chemical changes between the three samples (**Figure 7**). The shape of the thermogravimetric profile is identical for the expanded chitin regardless of drying method, but both differ from that of the neat flakes (**Figure 7A**). This is ascribed to mass and thermal diffusion limitations associated with the more compact flakes. The onset of thermal decomposition of all three samples occurs at 260 °C (**Figure 7B**), but the maximum decomposition rate for chitin flakes occurs at higher temperatures due to its more compact morphology.

[Place **Figure 7** here]

The increase in specific surface area is accompanied by an expected increase in the maximum uptake Cu by chitin. While the neat flakes uptake 13.8 ± 2.9 mg/g, the oven-dried foam uptakes 43.1 ± 1.9 mg/g and the lyophilized foam uptakes 73.1 ± 2.0 mg/g (**Table 1**). The increase in Cu uptake is more clearly illustrated by comparing the standard (**Figure 8A**) and linearized (**Figure 8B**) Langmuir adsorption isotherms. The maximum uptake is represented by the asymptotic limit in the standard isotherm and the inverse of the slope in the linearized isotherm. However, these differences in the uptake disappear when the Cu uptake is normalized by the surface area (**Table 1**). While the neat flakes uptake 10.5 ± 2.8 $atoms/nm^2$, the oven-dried foam uptakes 10.7 ± 0.4

atoms/nm^2, and the lyophilized foam uptakes 9.4 ± 0.3 atoms/nm^2 (**Table 1**). This suggests that the surface of the expanded chitin is chemically similar to that of the initial chitin flakes, which agrees with spectroscopy and thermogravimetric observations.

[Place **Figure 8** here]

FIGURE AND TABLE LEGENDS:

Table 1. **Summary of material properties**. Chitin foams have lower crystallinity and crystal size relative to neat chitin flakes. However, the specific surface area and Cu uptake of the chitin foams are proportionally higher than that of the neat chitin flakes.

Figure 1. Chemical structure. (**A**) **cellulose,** (**B**) **chitin,** (**C**) **chitosan.**

Figure 2. Preparation of expanded chitin foam. (**A**) The initial chitin in LiCl/DMAc solution. (**B**) The addition of the NaH/DMAc slurry. (**C**) The chitin foam after addition of water. (**D**) The chitin foam as extracted from the reaction flask. (**E**) The chitin foam during washing with water.

Figure 3. Photographs and micrographs of neat flake and expanded chitin. (**A**) The photographs correspond to chitin in its neat flake form. The expanded foam form dried by two different methods— (**B1**) baking at 80 °C and (**C1**) lyophilizing. (**A2,A3**) The scanning electron micrographs correspond to two magnifications of chitin in its neat flake form. The expanded foam form dried by two different methods—(**B2,B3**) baking at 80 °C and (**C2,C3**) lyophilizing. Note the more compact form of the neat flakes relative to the expanded foam.

Figure 4. X-ray diffractograms of neat and expanded chitin. The figure shows the diffractograms of chitin in its neat flake form and in its expanded foam form dried by two different methods—baking at 800 °C and lyophilizing. All three diffractograms are normalized to the maximum intensity of reflection at 19.3 °, which corresponds to plane (110). Note the general widening of peaks in the expanded foams relative to the neat flakes.

Figure 2. N_2 adsorption isotherms and BET plots. (**A**) N_2 adsorption isotherms of chitin in its neat flake form and in its expanded foam form dried by two different methods—baking at 80 °C and lyophilizing—for partial pressures in the BET range. (**B, C**) BET plot for the same materials and the range of partial pressures. The specific surface areas are proportional to the inverse of the sum of intercept and slope of the lines in the BET plots.

Figure 6. ATR IR spectrograms of neat and expanded chitin. The figure shows the IR spectra of chitin in its neat flake form and in its expanded foam form dried by two different methods—baking at 80 °C and lyophilizing. The differences in the spectra are minimal and suggest no significant chemical changes between neat flakes and expanded foam chitin.

Figure 7. Thermogravimetric profiles of neat and expanded chitin. The figure shows the integral (above) and differential (below) thermogravimetric profiles of chitin in its neat flake form and in

its expanded foam form dried by two different methods—baking at 80 °C and lyophilizing. The onset of thermal decomposition of all three materials is at 260 °C, but flakes decompose over a longer temperature range relative to the foams.

Figure 8. Standard and linearized (B, C) Cu adsorption isotherm. (**A**) The Cu adsorption isotherms of chitin in its neat flake form and in its expanded foam form dried by two different methods—baking at 80 °C and lyophilizing. Each data point is the average of three measurements and the error bars represent two standard deviations. Error bars for the expanded foams are small and can only be seen in (**C**). The solid lines show the best fit Langmuir adsorption isotherms. The maximum uptake is the asymptotic value in the standard adsorption isotherm and the inverse slope in the linearized ones. Expanded chitin shows a higher Cu uptake than that of chitin flakes by at least a factor of 4.

DISCUSSION:

The proposed method for chitin foam fabrication allows for the production of such foams without the need for specialized equipment or techniques. Production of the chitin foam relies on the suspension of sodium hydride within a chitin sol-gel. Contact with water from the atmosphere induces gelling of the chitin matrix and evolution of hydrogen gas by decomposition of the sodium hydride. Therefore, the critical steps of the preparation are (1) formation of the sol-gel, (2) introduction of the sodium hydride in anhydrous conditions, and (3) the reaction of atmospheric water with the chitin sol-gel and sodium hydride suspension.

Two important limitations arise from the third step. First, the process scales up poorly. The chitin sol-gel is a highly hygroscopic and readily absorbs moisture, but as the reaction volume increases, water diffusion limitations may prevent gelling. In fact, we observed that doubling the reaction volume increased the gelling time from days to weeks. Second, the process relies on atmospheric moisture. Local climate and seasonal weather will cause variations in the gelling time. A possible modification to the procedure is to use Schlenk techniques to maintain the reaction atmosphere air and moisture free, and then gradually add water to the chitin sol-gel and sodium hydride suspension. However, such a change requires resources and skills that would limit applicability.

Both the crystallinity index and crystal size reported above are only semi-quantitative estimates. The crystallinity index was calculated as described by Focher, et al.[29], and is therefore not a true crystallinity fraction. It was not obtained by comparing peak areas to those of standards of known purity. Similarly, use of the Scherrer equation to obtain crystallite size from the line broadening only provides estimates. Other phenomena, such as non-uniform strain, can also contribute to line broadening[34]. For this reason, it is more appropriate to focus on trends rather than the absolute values of crystallinity index and crystallite size. As recommended elsewhere, those values are reported without associated errors or variances[34].

Calculating specific surface areas by applying the BET equation to N_2 physisorption isotherms requires thorough drying and degassing of samples prior to the analysis. The presence of moisture and adsorbates on the sample will alter specific area measurements in two ways: (1) by blocking and lowering the effective number of vacant adsorption sites, and (2) by desorbing volatiles, increasing the measured pressure above the sample, and lowering its apparent adsorption. To prevent these errors, carbon and oxide samples are typically degassed at temperatures near 300 °C under flowing N_2 or vacuum for at least 1 h. Although structurally robust, chitin will thermally decompose under such conditions (**Figure 6**). Instead, specific surface area measurements of expanded chitin foams were most reliable for samples degassed at 50 °C under flowing N_2 for 1 week immediately after oven drying or lyophilizing.

Conducting adsorption isothermal experiments is routine, but the specific protocols vary greatly based on the adsorbent, solution, mixing method, available instruments, and convenience. For that reason, this study includes a detailed protocol based on a procedure for waste water analysis[28]. The adsorption of Cu on chitin is low relative to other adsorbents, such as carbons. Chitin requires high Cu concentrations in the range of 100–500 mg/L in order to reach saturation[35]. However, the colorimetric bicinchoninate method has a Cu detection ceiling of only 5 mg/L[27]. This means that aliquots had to be diluted 100 times for their Cu concentration to be measurable by the instrument. Dilutions can introduce significant experimental error into measurements, so the dilution and measurements were repeated three times per sample. Using a graduated cylinder to perform the dilutions, the observed variance in the measured concentrations were low—less than 3.7 % for low Cu concentrations and less than 0.35% for high Cu concentrations. The variance could be decreased by using volumetric flasks to perform the dilution. In addition, it is important to minimize the headspace during the adsorption experiments. Any adsorbent that adheres to the container walls above the liquid line will not equilibrate with the solution and will induce error in the experiment. This can be prevented by placing the containers at a 15° angle relative to the orbital plane of the shaker, and routinely shaking the containers by hand to dislodge any adsorbent adhered to the inner walls.

The Langmuir model for isothermal, non-dissociative adsorption assumes that (1) the analyte adsorbs in a single layer, (2) adsorption sites are energetically equivalent and can contain a single analyte molecule or ion, and (3) adsorbed molecules or ions do not interact with one another. The collected Cu adsorption data fits the Langmuir model and validates these assumptions. However, we used refined chitin harvested from a single species as the starting material. Using lower purity chitin, or chemically modifying the surface[17,36], can result in greater morphological and energetic variation between adsorption sites, which would call for a different adsorption model.

ACKNOWLEDGMENTS:

The research was sponsored by the Combat Capabilities Development Command Army Research Laboratory (Cooperative Agreement Number W911NF-15-2-0020). Any opinions, findings and conclusions, or recommendations expressed in this material are those of the authors and do not necessarily reflect the views of the Army Research Lab.

We thank the Center for Advanced Materials Processing (CAMP) at Montana Technological University for the use of some of the specialized equipment required in this study. We also thank Gary Wyss, Nancy Oyer, Rick LaDouceur, John Kirtley, and Katherine Zodrow for the technical assistance and helpful discussions.

DISCLOSURES:

The authors have nothing to disclose.

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Figure 1 - Structures

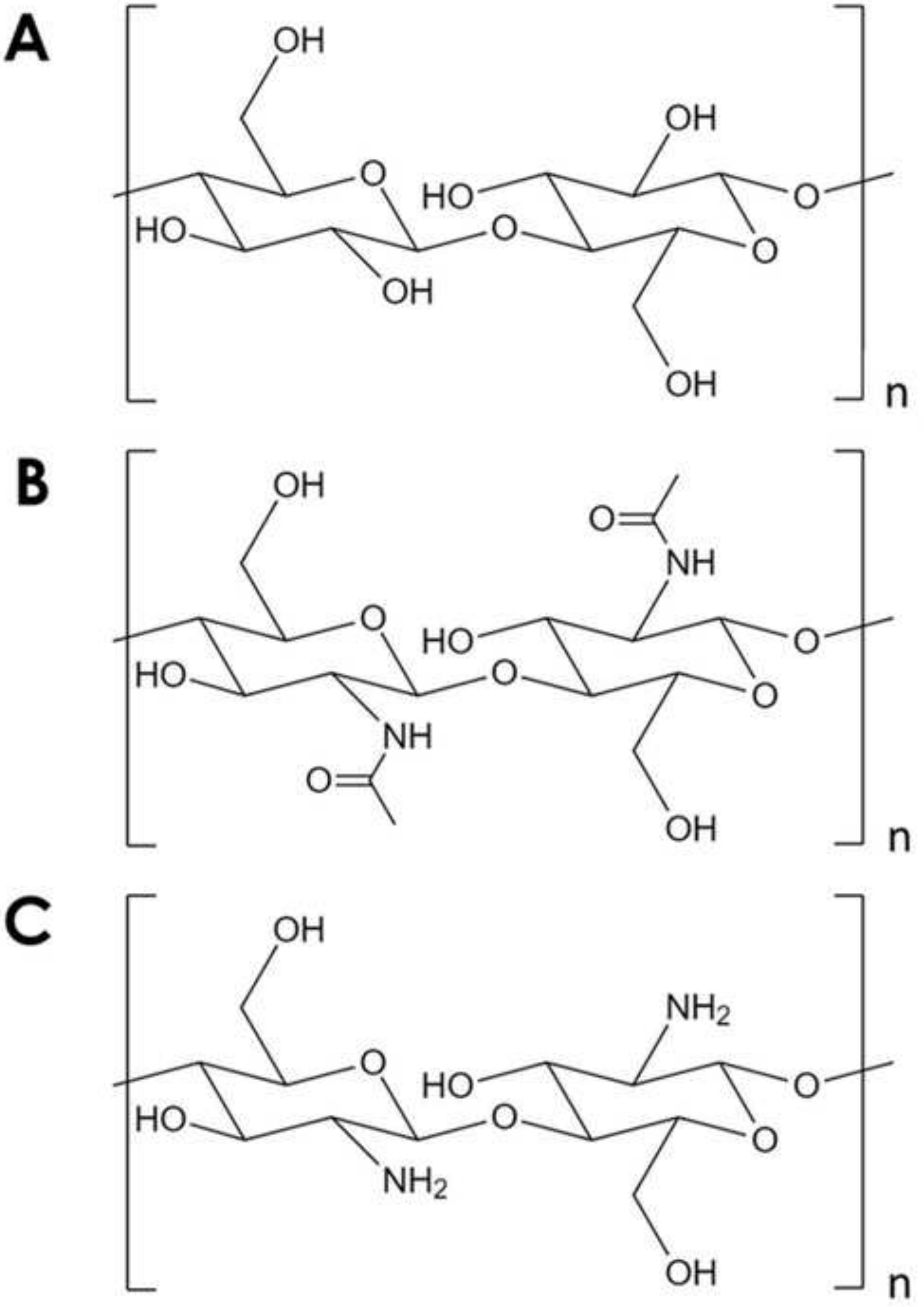
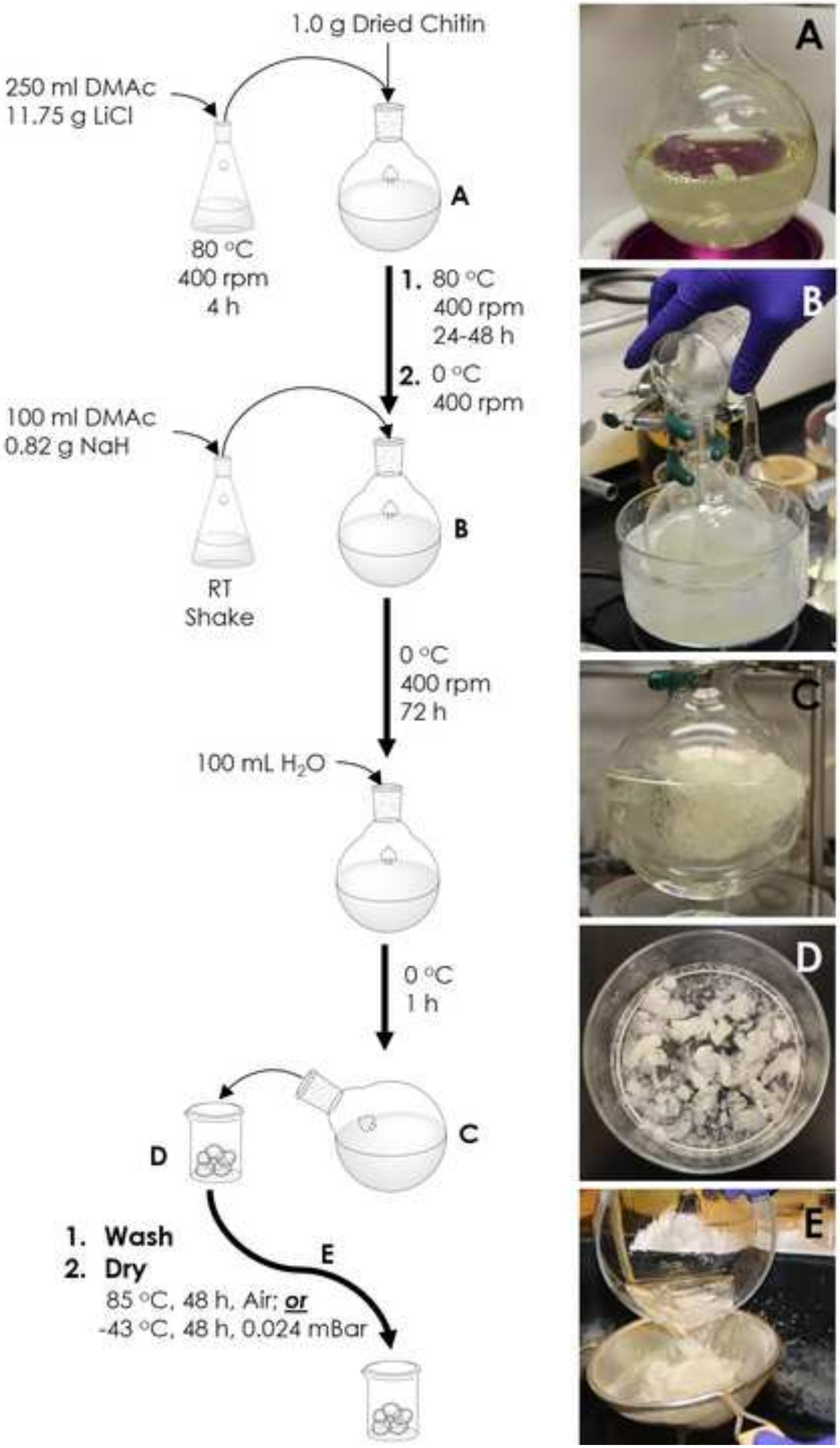
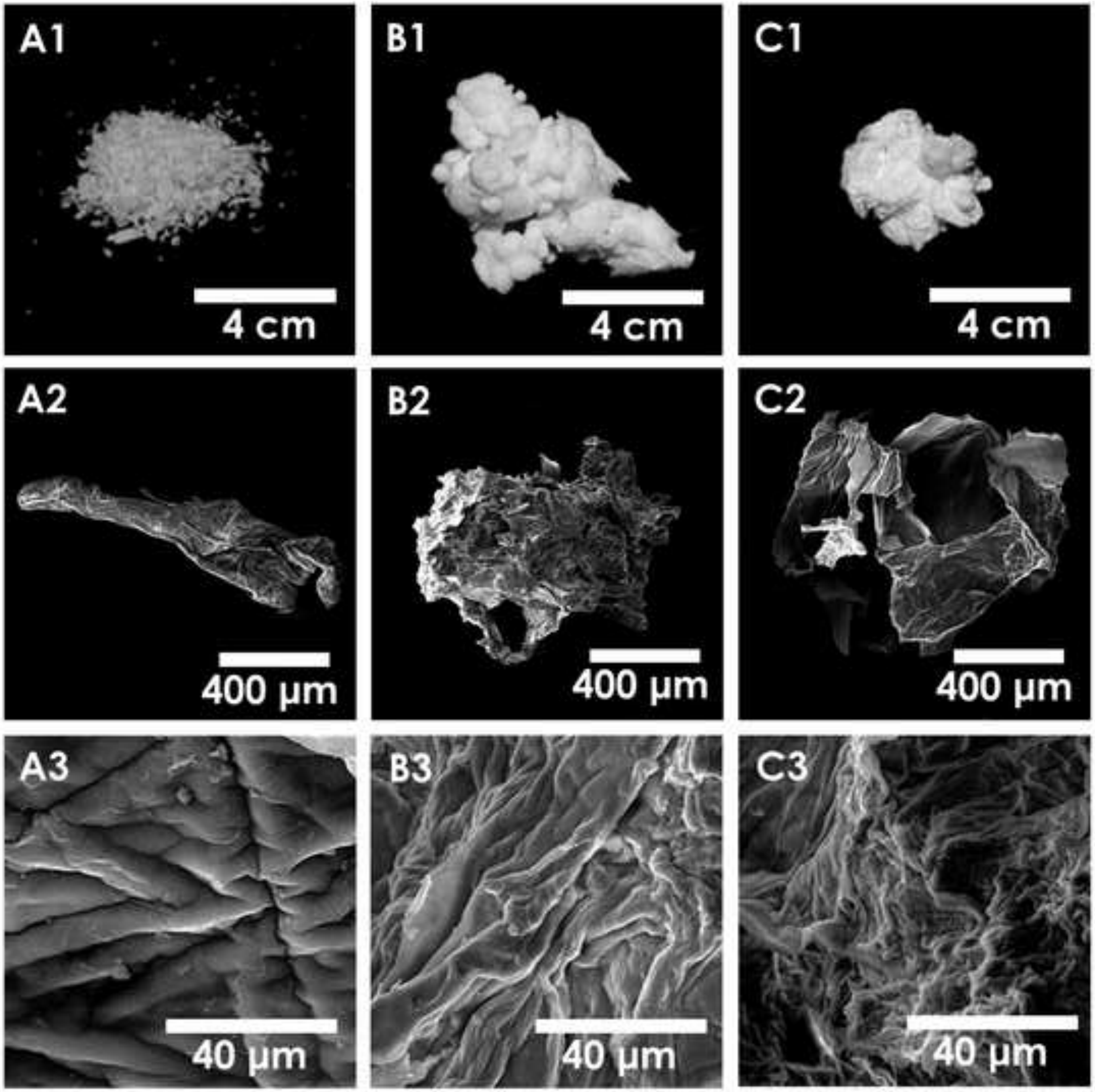


Figure 2 - Graphical method




A1
4 cm
B1
4 cm
C1
4 cm
A2
400 µm
B2
400 µm
C2
400 µm
A3
40 µm
B3
40 µm
C3
40 µm

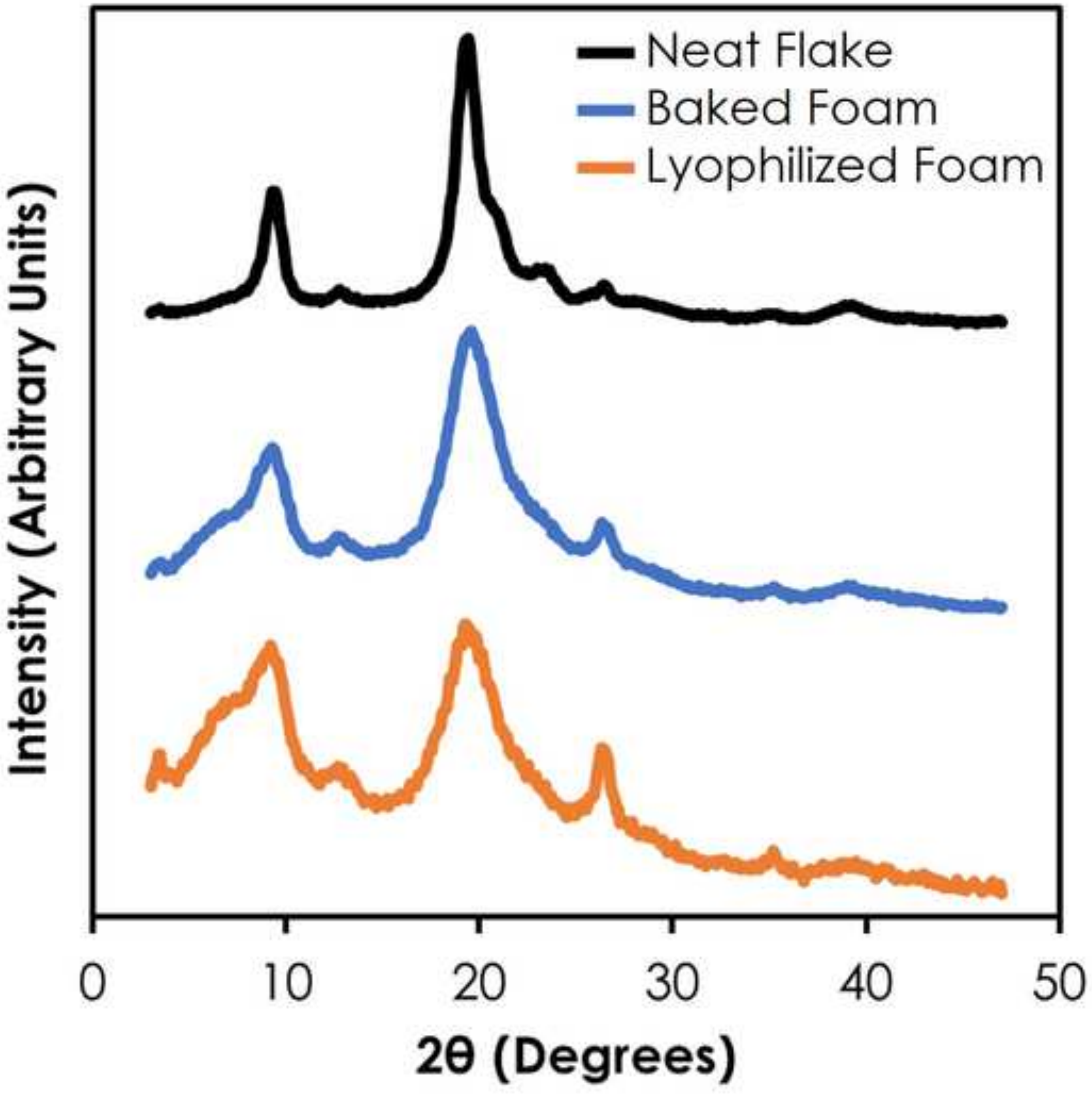
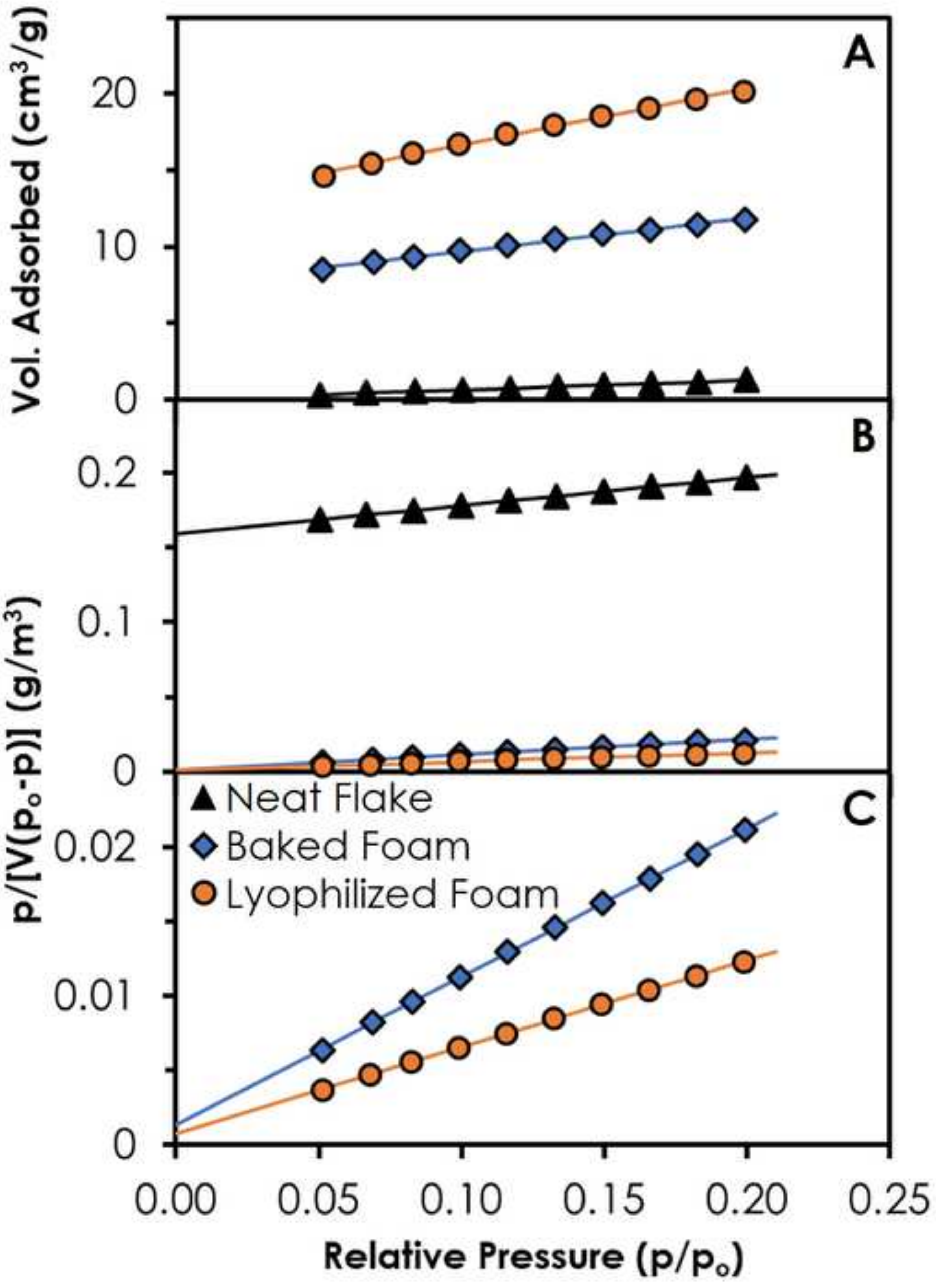
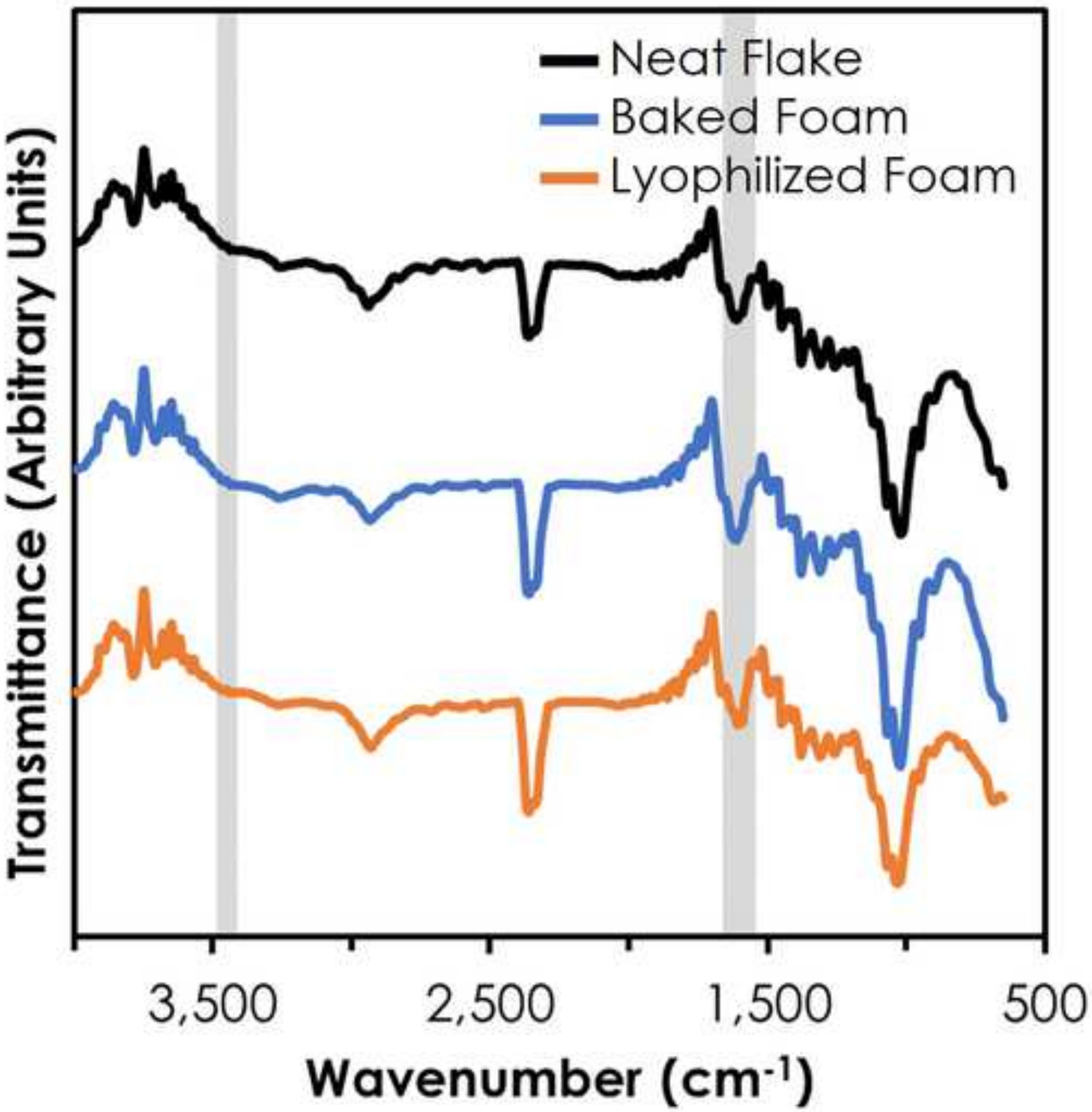
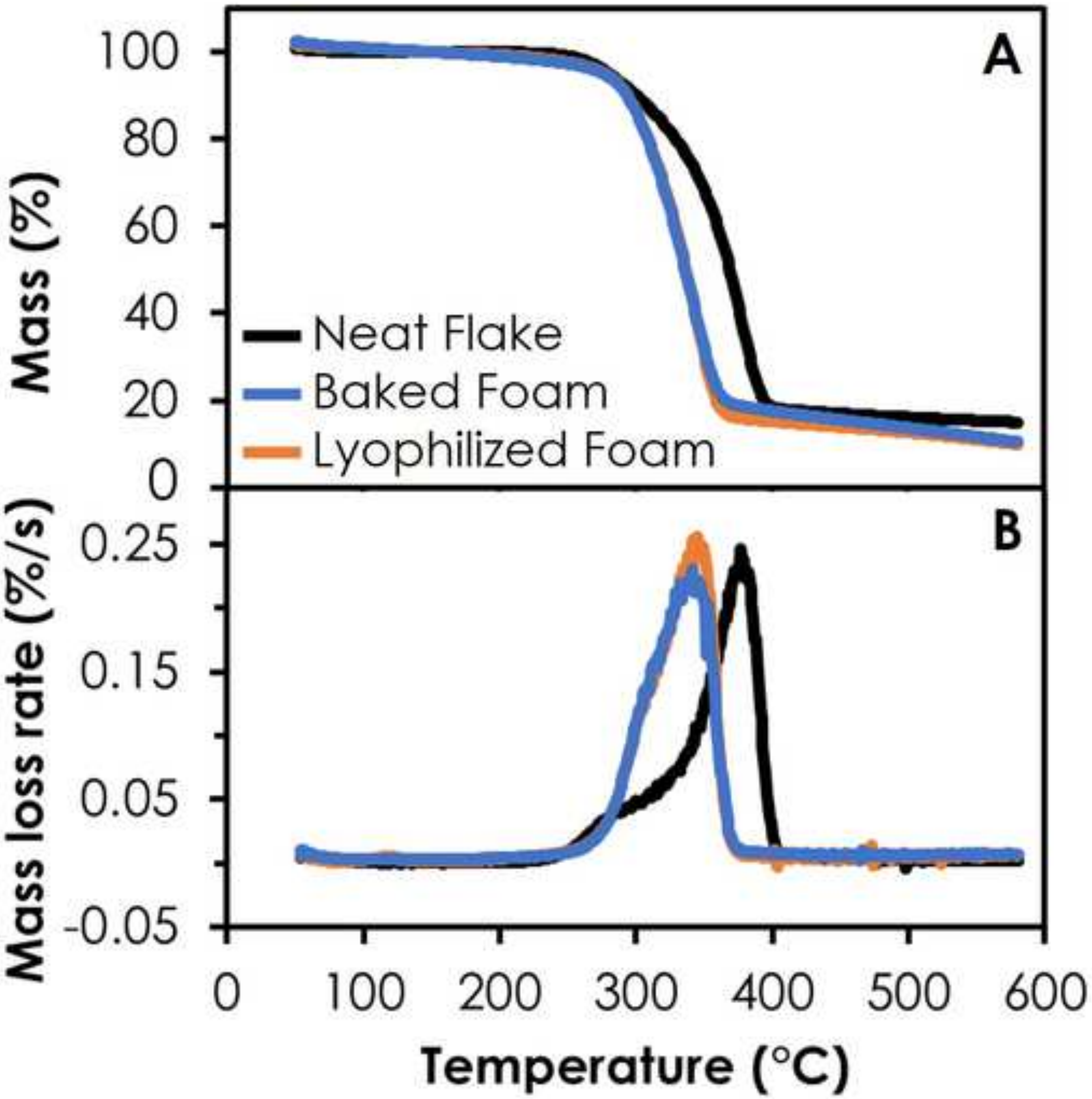

Neat Flake
Baked Foam
Lyophilized Foam
Intensity (Arbitrary Units)
0
10
20
30
40
50
2θ (Degrees)

Figure 5 - N2 sorption isotherm and analysis




Neat Flake
Baked Foam
Lyophilized Foam
Transmittance (Arbitrary Units)
3,500
2,500
1,500
500
Wavenumber (cm-1)


A
B
Mass (%)
100
80
60
40
20
0
Neat Flake
Baked Foam
Lyophilized Foam
Mass loss rate (%/s)
0.25
0.15
0.05
-0.05
0
100
200
300
400
500
600
Temperature (°C)

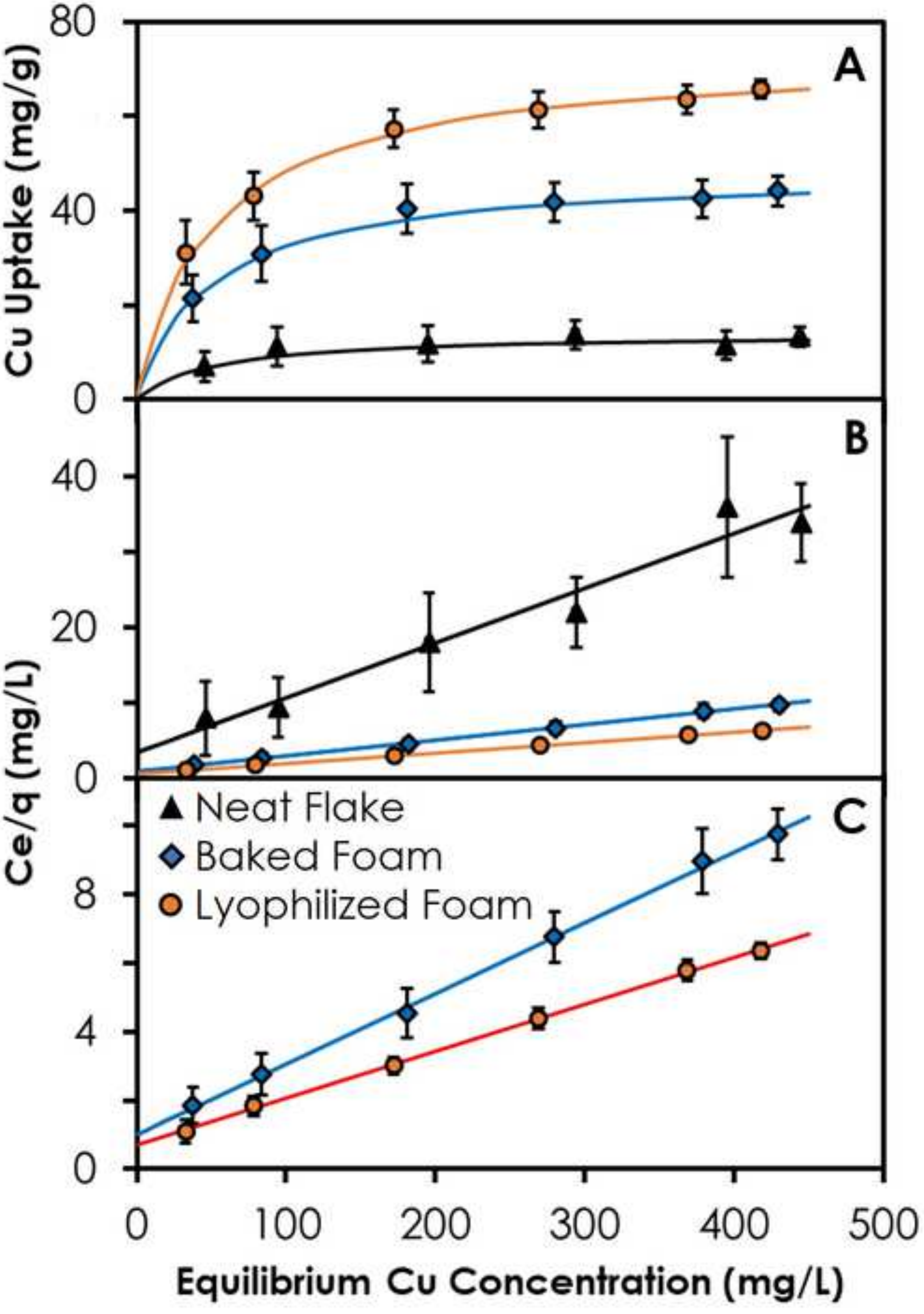

80
40
0
Cu Uptake (mg/g)
A
B
40
20
0
Ce/q (mg/L)
C
▲ Neat Flake
◆ Baked Foam
● Lyophilized Foam
8
4
0
0
100
200
300
400
500
Equilibrium Cu Concentration (mg/L)

Table 1 - Material property summary

	Unit	Neat Flake	Baked Foam	Lyophilised Foam
Crystallinity	%	88	74	58
Crystal size	nm	6.5	4.4	4.4
Surface Area	m^2/g	12.6 ± 2.1	43.1 ± 0.2	73.9 ± 0.2
Cu Uptake	mg/g	13.8 ± 2.9	48.6 ± 1.9	73.1 ± 2.0
Cu Uptake	$atom/nm^2$	10.5 ± 2.8	10.7 ± 0.4	9.4 ± 0.3

Name of Material/ Equipment	Company	Catalog Number
Ammonium bicarbonate	Sigma-Aldrich	9830
Chitin	Sigma-Aldrich	C7170
Colorimeter	Hanna Instruments	HI83399-01
Copper High Range Checker	Hanna Instruments	HI702
Copper nitrate hydrate	Sigma-Aldrich	223395
Dimethylacetamide (DMAc)	Sigma-Aldrich	271012
IR Spectrophotometer	Thermo Nicolet	Nexus 670
Lithium chloride	Sigma-Aldrich	310468
N2 Physisorption Apparatus	Micromeritics	Tristar II
Nitric acid	BDH	BDH7208-1
Scanning electron microscope	Zeiss LEO	1430 VP
Sodium hydride	Sigma-Aldrich	223441
Thermogravimetric analyzer	TA Instruments	Q500
Water Purification System	Millipore	Milli-Q
X-Ray Diffractometer	Rigaku	Ultima IV

Comments/Description
NH4HCO3, ≥99.5 %
Pandalus borealis, practical grade
Photometer for wastewater analysis
Bicinchoninate colorimetric titration
Cu(NO3)2 · 2.5 H2O, 98 %
Anhydrous, 99.8 %
Fitted with an ATR cell
LiCl, ≥99 %
HNO3, 0.1 N
15 kV, secondary electron detector, 29-31 mm working distance
NaH, packed in mineral oil, 90 %
100 ml/min N2, 10 °C/min to 800 °C
Type A water (18 MΩ)
Cu K-α radiation, 8.04 keV

January 22, 2021

Dear Editor –

Thank you for taking the time to review our submission and provide us with timely feedback. The comments, concerns, and clarifications are sincerely appreciated. Please find our responses to specific comments below. We have addressed all of them to the best of our abilities, but we request some additional guidance on your third comment on RPM vs. xg for mixing processes.

In addition to the corrections listed immediately below, we made some additions and corrections to address the specific concerns of the reviewers. Most significantly, a new figure was added to include N_2 sorption data. As a result, the discussion of the specific surface area measurements was expanded. You can find more details in the three letters (below) we prepared to address the comments and concerns of individual reviewers.

Thank you again for the consideration and for accommodating our request of additional time.

Sincerely,

The Authors

Editorial Comments

1 Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

> The authors thank the editor for this reminder. The document has been proof-read again by all authors and all spelling and grammar issues encountered have been addressed.

2. Please revise the text to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.).

> The authors thank the editor for clarifying the preferences of the journal. All usage for personal pronouns has been removed.

3. Line 98/181: Please convert the stirring speed to centrifugal force (x g) instead of revolutions per minute (rpm).

> The authors thank the editor for comment but remain under the impression that only centrifugal separations must be reported in *xg*. Part B.2 of the protocol instructions in the JoVE template only mentions using *xg* when describing centrifugal force. Other recent articles in JoVE (DOI: 10.3791/56078, DOI: 10.3791/60768, DOI: 10.3791/61340) report stirring and mixing speed in RPM. Please advise.

4. Line 103/190: Please use abbreviated forms for durations of less than one day when the unit is preceded by a numeral for units of time. Examples: 5 h, 10 min, 100 s.

Thank you for the correction. These discrepancies have been corrected.

5. Line 106: Please mention if there is a specific size for the magnetic stir bar.

We thank the editor for pointing out this oversight on our part. The magnetic stir bars used were always 50 mm x 8 mm. This is now indicated in the procedure in steps 1.1.1 and 1.2.2.

6. Line 108: Please mention how is the flask filled with Argon.

We thank the editor for the comment. This is an error on our part and we sincerely appreciate you bringing it to our attention. The requirement of the inert Ar atmosphere is a remnant from an earlier version of this manuscript. During initial tests we believed the process to be very air and moisture sensitive. That was ultimately not the case and there is no need for the strict air- and moisture-free techniques in the synthesis. Accordingly, the text of this step has been revised to:

1.2.3 Cap the flask with a rubber septum and place it on a stirring heat block. Pierce the septum with a needle and leave it place to allow the flask to vent. Heat the block to 80^{o}C and stir the mixture at 400 rpm until all chitin is dissolved (24-48 h).

7. Line 135: Please mention if there is a specific Chitin sol-gel: NaH slurry ratio or the prepared solutions are added completely.

We thank the editor for requesting this clarification. All the NaH slurry must be added to the chitin sol-gel. This is indicated in steps 1.4 and 1.4.1 by the inclusion of the word *all*.

8. Line 190/193/197: Please use standard abbreviations when the unit is preceded by a numeral. Abbreviate liters to L to avoid confusion. Examples: 10 mL, 8 µL.

We thank the editor for pointing out these issues. We have corrected the volume units on lines 190, 193, and 197 (in addition to others on lines 89, 95, 117, 127, 141, 168, 170, 173, 175) to conform to the format of JoVE.

9. Please highlight up to 3 pages of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol. Remember that non-highlighted Protocol steps will remain in the manuscript, and therefore will still be available to the reader.

Thank you for the additional direction on this manner. The most important part of the synthesis process is now highlighted.

10. Figure 3: Please provide the details of the scanning electron microscopy settings in the Representative Results.

We thank the editor for this comment and apologize for the omission. The following text has been to the Representative Results section, right after the micrographs are described.

The samples were sputter coated with gold before imaging with a secondary electron detector, with a 15 kV accelerating voltage, and at a working distance in the range 29-31 mm.

In addition, the instruments operating conditions have been added as a comment to the table of materials.

11. Figure 4/5/6/7: Please consider placing the units of the X and Y-axis within parentheses.

> We thank the editor for this comment and apologize for the oversight. All units on X and Y axis labels now match JoVE's prescribed formatting.

12. Figure 7: Please consider reducing the size of the symbols used in the figure to make all the error bars visible (especially in Figure 7B, the error bars of the baked foam and lyophilized form are not visible)

> Thank you for pointing this out. Decreasing the marker size in in the figure to show the error bars would render the markers to small to distinguish. Instead, a third panel is now included (C) showing a smaller portion of Y-axis. The error bars can be seen in panel C.

January 22, 2021

Dear Reviewer No. 1

Thank you for the comments, suggestions, and interest. The provided references were especially helpful. We have addressed your concerns and our specific responses are below. Please note that due to the focus of the journal on methods, some of your suggestions were incorporated by modifying the introduction and not the discussion.

Kind regards,

The Authors

Major Concerns

1. After the adsorption of copper by the chitin foam material, can the material adsorb copper again? Can the material achieve the effect of repeated adsorption?

> We thank the reviewer for the comment and appreciate the interest. The desorption and regenerability of the chitin materials was not tested in this study, but it is an aspect of current research by the group. Initial results suggest that Cu can be partially removed in acidic media, but the effectiveness of the chitin upon reuse is still being investigated. Because that research is ongoing and the results have yet to be reproduced and validated, we have not included details in this paper.

2. Although the material already has a good adsorption performance, and the error in the adsorption process is clearly explained, does the author consider further innovations to further improve the adsorption effect of the material on copper?

> We again thank the reviewer for the comment and sincerely appreciate the interest. This technique for expanding chitin into foams was a serendipitous discovery the lead author made while attempting to functionalize chitin. Indeed, it appears that simply expanding chitin does not increase the inherent adsorption capacity of chitin by surface area, so functionalizing the surface and modifying its chemical make-up is a strategy the group is currently pursuing. Because that work has not yet been completed, we avoided any mentions of it.

3. The partial analysis of adsorption mechanism in this paper is not thorough enough. It is hoped that the author can further explain the adsorption mechanism from the perspective of the interaction between expanded foam chitin and copper. The author may refer to the following references and cite them in the references：

a. Y. He, H. Tian, A. Xiang, H. Wang, J. Li, X. Luo, A.V. Rajulu, Fabrication of PVA Nanofibers

Grafted with Octaamino-POSS and their Application in Heavy Metal Adsorption, Journal of Polymers and the Environment, (2020). DOI: 10.1007/s10924-020-01865-x

b. H. Tian, L. Yuan, J. Wang, H. Wu, H. Wang, A. Xiang, B. Ashok, A.V. Rajulu, Electrospinning of polyvinyl alcohol into crosslinked nanofibers: An approach to fabricate functional adsorbent for heavy metals, J Hazard Mater, 378 (2019) 120751. DOI: 10.1016/j.jhazmat.2019.120751

> We thank the reviewer for their encouragement and the very insightful references that are now included in the paper. We have expanded on the interactions between chitin and metal ions in the introduction, which now includes the following text in lines 66-71:
>
> *In addition, the abundance of amine groups make chitin an effective adsorbent for metal ions. [ref 17] However, the protonation of the amine groups under acidic conditions reduces the metal adsorption capacity of chitin. [ref. 18] A successful strategy is to introduce adsorption sites more resistant to protonation. [19, 20] Instead, herein is described a simple method to increase the specific surface area and, therefore, the number of adsorption sites in chitin.*
>
> However, the journal format limits the discussion section of the paper to six paragraphs strictly focused on the methods. Because of the strict focus, the discussion was not modified.

4. Does the photographs and micrographs of chitin foam material change after adsorption? If the author can add the photographs and micrographs of absorbed material to Figure 3, it may give a better description of the adsorption situation.

> We thank the reviewer for the interest and the suggestion. Our initial intent was to recover the chitin after adsorption and characterize the Cu structure by Diffuse Reflectance UV-Visible spectroscopy. Unfortunately, the adsorption tests used a very small amount of chitin (50 mg) which we could nor recover successfully.

Minor Concerns:

There are many careless errors and grammatical errors in the article, which are as follows:
1) Page 3, line 124-131, every paragraph should be followed by a period.

2) Page 4, line 141-142, the paragraph 1.5.1 should be aligned.

3) Page 5, line 183, the end of paragraph 2.2.3 lacks a period.

> We thank the reviewer for noticing our oversight. We have corrected these errors.

January 22, 2021

Dear Reviewer No. 2

Thank you for the taking the time to review our work so carefully. We sincerely appreciate your comments and suggestions, most of which we gladly incorporated into the paper. You will find our detailed response below.

The most significant change is the inclusion of a new figure showing the N_2 sorption data. As a result, the discussion of the specific surface area measurements was expanded. More details are below. Please note that due to instrument limitations we do not have the full adsorption isotherm and cannot make inferences on the pore structure. Instead, we only have data in the BET region. For that reason, we have removed all definitive mentions of an increase in porosity.

Thanks again. Sincerely,

The Authors

Major Concerns

1. **Step 1.3.2 - why is a volumetric flask used to make this mixture? It does not appear to be essential (in terms of making an exact volume) at any point;**

   We thank the reviewer for pointing out this mistake. A volumetric flask is indeed not needed, and it was an Erlenmeyer flask that was used. The text was changed accordingly.

2. **Step 2.1 - the suggested mg amounts do not equate to the final desired concentrations (in mg/L). For example, the first preparation of 50 mg/L is suggested as 90 mg in 500 mL. This would be 90 mg/0.5 L which is the same as 180 mg/1 L. Perhaps you were trying to calculate mM?;**

   We thank the reviewer for taking the effort to check our numbers. However, the given numbers are correct. Note that the solutions are prepared from $Cu(NO_3)_2{\cdot}2.5H_2O$, which has a molar mass of 232.6 g/mol. As such, 90 mg of $Cu(NO_3)_2{\cdot}2.5H_2O$ contains only 25 mg of Cu and its dissolution in 500 mL of water results in a Cu concentration of 50 mg/L. We do understand the source of confusing, so we have modified the text in step 2.1 to show the difference in molar weight between the solute of interest (Cu^{2+}) and the precursor salt ($Cu(NO_3)_2{\cdot}2.5H_2O$)

   2.1. Prepare 500 mL stock solutions of aq. Cu^{2+} (MW 63.5 g/mol) at concentrations 50, 100, 200, 300, 400, and 450 mg/L. To do this, charge six containers with 90, 180, 360, 540, 720, and 810 mg of $Cu(NO_3)_2{\cdot}2.5\ H_2O$ (MW 232.6 g/mol), add 500 mLl of 18 MΩ water, cap the container, and shake to dissolve the solids.

3. **Step 2.3.2 - how precise do the dilutions need to be? If they need to be precise, wouldn't it be best to dilute using a 100 mL volumetric flask to ensure proper dilutions?;**

   We thank the reviewer for comment. In our experience, the dilutions do need to be precise and using a volumetric flask does increase precision. However, using a graduated cylinder instead, we found the measurement-to-measurement variance of triplicates to be less than 3.7 % for low Cu concentrations and less than 0.35% for high Cu concentrations. We deemed these variances to be an acceptable tradeoff for the increase in analysis speed. We have updated our discussion to include this information. The discussion of the Cu adsorption measurement no includes the text:

Using a graduated cylinder to perform the dilutions, the observed variance in the measured concentrations were low—less 3.7 % for low Cu concentrations and less than 0.35% for high Cu concentrations. The variance could be decreased by using volumetric flasks to perform the dilution.

4. **Overall: how do you know that the increase in surface area is not proportional to the smaller particles? More small particles = higher surface area. Do you think most of the increase in surface area comes from the expansion of the pores? I think your SEM and uptake results at least somewhat support this, but it's hard to determine exactly.;**

   We sincerely thank the reviewer for bringing this up. Unfortunately, our N_2-physisorption apparatus can only collect isotherms for partial pressures p/po 0.05 to 0.25---the BET region---and not full isotherms. As such, we have no quantitative way of comparing the porosity (pore size distribution, pore volume, etc) of neat and expanded chitin samples. We cannot rule out the possibility of small particles being the main contributor to the increased surface area, so we have removed all definitive references to increased porosity. Instead, we have updated the discussion of surface areas to include the following text:

   The changes in crystallinity index, crystallite size, and specific surface area indicate that the expansion process generates an open structure with smaller crystal domains that the material either (1) forms a more open and porous structure, or (2) is degraded into smaller particles. The micrographs in figure 3 suggest the former, but the latter cannot be ruled out without a thorough pore-size distribution analysis.

5. **Copper uptake figure - the color of the lyophilized foam circle in the legend does not match the red used with the actual data.**

   Thank you for pointing out this error. The color of the marker in the legend has been corrected.

6. **Are there error bars on the second part of that graph for the expanded foams? The error bars on the neat flakes are quite large, so it is interesting not to see them on the expanded foams. Are they so small they are not seen, or was this measurement not performed in triplicate?**

   We thank the reviewer for noticing this. All measurements were performed in triplicate, but the variance in the expanded samples decreased significantly when the in the linearized graph when the equilibrium concentration (Ce) was normalized by the uptake (q). Note that q depends on Ce (q = V*(Co-Ce)/m), so its not surprising that the variance would increase for the neat chitin (low adsorption and Ce close to Co) and decrease for the expanded foams (high adsorption and Ce different from Co). To avoid confusion, a third panel with a magnified Y-axis was added to the graph. The error bars of the expanded foams can be clearly seen in it.

7. **Where are the nitrogen sorption isotherms? Their shape could help answer if the increase in surface area is due to expansion of pores or just reduction in particle size.**

   We sincerely thank the reviewer for the suggestion, and we have included the N2 adsorption isotherms as figure 5. However, please note that due to instrument limitations we could only collect data for partial pressures p/po between 0.05 and 0.25. Because we cannot produce the full adsorption isotherms, we cannot quantify the changes in porosity between samples. As such, we have removed all definitive mention of increased porosity from the manuscript and focus solely on increases in specific surface area.

Minor Concerns:

1. **Step 1.2.3 - does the flask need to first be evacuated and filled to create an inert atmosphere? If not (air is ok in the flask), why should it be filled with Ar at this step?;**

   Thank you for the comment. This is an error on our part and we sincerely appreciate you bringing it to our attention. The requirement of the inert Ar atmosphere is a remnant from an earlier version of this manuscript. During initial tests we believed the process to be very air and moisture sensitive. That was ultimately not the case and there is no need for the strict air- and moisture-free techniques in the synthesis. Accordingly, the text has been revised to:

   1.2.4 Cap the flask with a rubber septum and place it on a stirring heat block. Pierce the septum with a needle and leave it place to allow the flask to vent. Heat the block to 80^{o}C and stir the mixture at 400 rpm until all chitin is dissolved (24-48 h).

2. **Step 1.1.2 and 1.2.3 - how is 80 ℃ heating ensured if the flask is capped? Is this in an oil bath? Pictures show a bath of some type.**

   Thank you for the comment and apologies for the oversight.

   In step 1.1.2, the temperature of the mixture controls the hot plate. To do so, a heating stir plate with a built-in temperature probe and PID controller was used. The flask is capped with a rubber septum and a temperature probe is passed through it. To reflect this more clearly, the text in step 1.1.2 has been revised to:

   1.1.2. Cap the flask with a rubber septum and place it on a heating stir plate. Place a temperature probe through the septum into the mixture. Stir the mixture at 400 rpm and 80 °C until all LiCl is dissolved (~ 4 h)

   In the revised text, we leave the nature of the temperature probe and controller ambiguous, because our specific set-up is not necessary. The procedure can carried out with a thermometer and standard hot plate.

   In step 1.2.3, the flask containing the mixture is placed in an aluminum block fitted on the same heating stir plate described above. The temperature of the heating block is monitored and controlled. To reflect this more clearly, step 1.2.3 has been revised to:

   1.2.3 Cap the flask with a rubber septum and place it on a stirring heat block. Pierce the septum with a needle and leave it place to allow the flask to vent. Heat the block to 80^{o}C and stir the mixture at 400 rpm until all chitin is dissolved (24-48 h).

3. **Step 2.2.1 - are the containers capped so that they do not spill during the shaker step?**

   Thank you for noticing our omission. The containers are indeed capped to avoid spills. The text in step 2.2.1 has been revised to:

   1.1.1. Transfer 100 mL of each stock solution to a 100 mL container so the headspace is *minimal. Add 50 mg of ground chitin to each container and then cap them.*

4. **At least 1 more article from recent years (2017+) citing the usefulness of porous chitin foam for the suggested applications (as adsorbents or as supports) should be cited. I see at least 3 articles cited to reference the potential application of these materials, but only one is from 2018. Many of the other articles, appropriately, are synthesis or analysis articles. Most of these are not from the last 5 years, and when showing relevance of this work, there should be some precedence in the recent literature.**

   We thank the reviewer for this suggestion. We have enriched the introduction and discussion with several references from 2019, 2020, and 2021 on chitin flakes, fibers, and foams used as scaffolds for tissue growth, fibers in composite materials, and supports for catalysts and adsorbents.

January 22, 2021

Dear Reviewer No. 3

Thank you for the kind comments and useful suggestions. We have incorporated most into the paper and our detailed responses to each are below. The formatting of the methods section was left unchanged, as it is prescribed by the journal to make protocols as clear as possible. Otherwise, the most significant change is the inclusion of a new figure showing the N_2 sorption data. The new graphs is accompanied by an expanded discussion of the specific surface area measurements. Please note that due to instrument limitations we do not have the full adsorption isotherm. Instead, we only have data in the BET region.

Sincerely,

The Authors

Minor Concerns

1) **The procedure is very thorough but is presented in an uncommon format. The reviewer recommends the authors consider that the sections of the experimental section (i.e. 1.1, 1.2, etc.) be presented in paragraph format instead of short subsections (i.e. 1.1.1, 1.1.2, etc.)**

    Thank you for the feedback. We understand that this format of the procedure is unusual in research papers, but it is prescribed by the journal to make the method as clear as possible.

2) **The reviewer is curious if the authors were able to successfully perform quantitative spectroscopic measurements using the intrinsic color of copper nitrate solutions or ammonia complexes in lieu of using a specified colorimetric method for measuring water samples (the bicinchoninate colorimetric titration). This may make the method more accessible without the need to purchase measurement kits and may have a lower extinction coefficient to allow for lower dilution factors in preparing appropriate concentrations for analysis.**

    Thank you for the comment and the useful suggestion. We agree that the Cu measurement could be made more accurate and less costly by other methods. However, we did not attempt any quantitative spectroscopic measurements of copper other than the bicinchoninate method. Our current reliance on it stems from a long history of extremely high Cu contamination of ground water in our community. The bicinchoninate method with pre-measured reagents is recommended by the US Environmental Protection Agency, so it is widely used by local environmental entities and the expertise was readily available. In the future we plan to use to elemental analysis by ICP-AES instead, which is newly available to us.

3) **Preparation of analytical standards for colorimetry should be made using volumetric glassware. The reviewer recommends specifying the use of 100 mL volumetric flasks and either adjustable micropipettes or 1 mL volumetric pipettes to prepare the diluted samples.**

    We thank the reviewer for this suggestion and apologize for the apparent carelessness. The suggestion would undoubtably lower the measurement-to-measurement variability and the magnitude of the error bars shown in figure 8. However, the version of the bicinchoninate method we use was developed as a field test for waste and ground water samples. It is prescribed to be used with marked vials and pre-measured reagent packets supplied by an accredited vendor.

4) **The reviewer would like to see the nitrogen adsorption isotherms since the resulting values were important to comparisons made in the paper and the surface area of the samples are integral to the thrust of the paper (making high surface area foams).**

   The authors thank the reviewer for this suggestion and apologize for the oversight. The adsorption isotherms for all three samples are now included as figure 5. Figure 5A shows the raw adsorption isotherm, Figure 5B shows the BET plot, and Figure 5C repeats the BET plot with a magnified Y-axis. In addition to the new figure, the discussion of representative results N_2 sorption has been expanded as follows:

   For all materials, the N_2 adsorption isotherms show the uptake volume to increase linearly with partial pressure in the range p/po = 0.05-0.25 (Fig. 5.A), as is expected of multilayer condensation of N_2. [ref. 31] However, the uptake volume is greatest for the expanded foams. The BET plot (Fig. 5.B, 5.C), show a positive linear correlation with partial pressure and positive intercept, indicating that the data is within the valid range of the BET equation.[ref. 32] As such, the specific surface area of the materials is proportional to the inverse of the sum of the slope and intercept of those lines.30 While the specific surface area of the neat flakes is 12.6 ± 2.1 m^2/g, that of oven dried foam is 43.1 ± 0.2 m^2/g, and that of the lyophilized foam is 73.9 ± 0.2 m^2/g. The changes in crystallinity index, crystallite size, and specific surface area indicate that the expansion process generates an open structure with smaller crystal domains that the material either (1) forms a more open and porous structure, or (2) is degraded into smaller particles. The micrographs in figure 3 suggest the former, but the latter cannot be ruled out without a thorough pore-size distribution analysis.

   Please note that due to instrument limitations, we could not collect the entire adsorption and desorption isotherms. Instead, we only have access to pressures p/po in the BET range (0.05 to 0.20).

5) **The reviewer recommends remaining consistent on unit spacing (spaces should exist between values, +/- symbols, uncertainties, and units), especially on lines 182, 304, 306, 310, 315, 319, and 320.**

   We thank the reviewer for the feedback. All issues noted have been corrected.

6) **The reviewer noticed that line 159 states that the baked samples were dried for 24 h and Figure 2 states that samples were dried for 48 h. Similarly, line 160 states that the lyophilized samples were dried for 48 h, however Figure 2 states that these samples were dried for 1 h.**

   We thank the reviewer for pointing out this discrepancy and sincerely apologize for our carelessness. Ultimately, all drying times, regardless of method, should read 48 h. The errors reflect earlier versions of method when we were still experimenting with drying times. We have changed the text in line 159 and figure 2 to reflect the correct drying time of 48 h.

7) **Line 365 should be changed from "Calculating specific surfaces areas…" to "Calculating specific surface areas…"**

   We again thank the reviewer for the comment. The mistake has been corrected.