1. **Final Report**

Scalable synthesis of Cellulose Acetate and Protein Amyloid Fibers Membranes to Clean up Energy Production Effluents

M. Pawar, R. Souchereau, and P.I. Gouma

Problem and Research Objectives:

Produced water is naturally occurring water that comes out of the ground during oil and gas recovery, whereas *flowback water* is waste water from hydraulic fracturing, an unconventional energy production process. In hydraulic fracturing million gallons of water per well are injected in the sub- surface at high pressure along with sand and other chemicals. The high-pressure mixture cracks the rock and releases oil or gas. *In the United States alone, 300,000 fracking wells generate 1.5 billion barrels of oil and as many barrels of produced water each year.* The terms flowback water and produced wastewater (together referred to as Energy-Production-Effluents - EPE) are defined as effluent that rises to the surface during the hydraulic fracturing process, containing BTEX (benzene, toluene, ethylbenzene, and xylene) compounds, heavy metals, radionuclides, sealants, microbes, and salts. This water is often put into open pits for evaporation and/or is eventually transported to disposal sites or cleaning facilities.

Objective I: **Clean up produced water from fracking**

There are a few pilot studies reported on the use of produced water for agricultural processes, including irrigation. Under the Clean Water Act's Subpart E of 40 CFR Part 435, wastewater from oil and gas facilities may be re-used in agriculture West of the 98th meridian if it contains less than 35ug/L of oil and grease. Chevron Texaco has been recycling treated produced water in Southern California selling it to farmers for irrigation of fruit trees and other crops. The quantity of recycled water is 21 million gallons a day and it is used to irrigate 45,000 acres of crops in Kern County farmlands. While this practice has been going on for quite a long time now and it has been deemed successful by the local governing agencies, *concerns have been raised by civic organizations.*

A potential solution of the energy effluent water problem described here would be to reclaim (and fully clean) water from fracking for irrigation purposes and to recycle water for use both for food (crops, animals, animal products) and energy production (biofuels and re-fracking). ***This requires effective management of the Food (agriculture) –Energy (fracking)- Water (produced water) cycle.***

Objective II: **Scalable process of Amyloid fibers as Universal Filtration Agents**

There are numerous challenges to be met as produced water is a complex fluid containing diverse components that are difficult to remove: inorganic compounds, such as salts; toxic organics (BTEX hydrocarbons; naphthalene, etc.); even biologicals and radionuclides, just to name a few. The specific treatments that are needed to be developed and the precise contaminants which need to be removed are being described in this work. Previously we have demonstrated photocatalytic technology that break down hydrocarbon pollutants in water. The approach followed here is complementary to our earlier work and involves using wheat flour to produce amyloid fibers as a

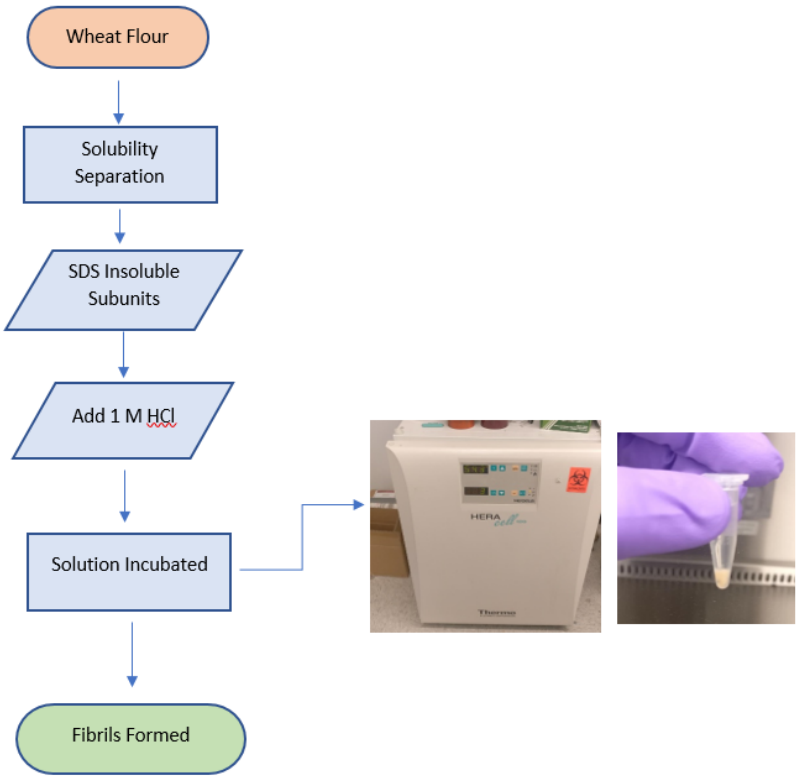
membrane component to treat produced water by capturing NORM and heavy metal contaminants, while also using remediated water to irrigate wheat crops in a closed loop operation.

The properties of amyloid fibrils, including high structural stability, nanoscale dimensions, ease of production, provide ample opportunities for technological uses. Mezzenga et al [1] incorporated amyloid fibrils into carbon membranes for water purification purposes. They presented amyloid fibrils as capable to capture heavy metal ions which cause disease in human body. They also proposed a method to recover precious metals recovered through amyloid water purification system. Another example of exploitation of amyloid's unique properties was given by Barrau and coworkers [2] by integrated amyloid nanowires in organic solar cells. Therefore, amyloid fibers have immense potential in terms of their utilization and hence economical and easy production methods for their formation are essential. This prompted us to investigate the synthesis of amyloid fibrils from wheat flour proteins and their use as water purification agents.

Methodology:

Amyloid fibrillation: Wheat flour contains different proteins that can be classified as Albumins, Globulins, Gliadins and Glutenins [3]. Using Osborne solubility rules [4], each group of proteins was separated out. The only proteins to form amyloid fibrils under acidic conditions were SDS-insoluble subunits of the Glutenins, meaning the aim of the separations was to isolate those subunits. The desired amount of wheat flour to be used was arbitrary, but each of the separation steps followed a 0.4 mL of solution per 100 mg of wheat flour initially added. Weighed desired amount of wheat flour and added to Eppendorf tube. 2% (w/v) NaCl solution was first prepared and subsequently 0.4 ml per 100 mg of initial wheat flour was added to the Eppendorf tube. The tube swapped off 5 minutes of pulsing and 5 minutes of vortex, for 30 minutes. Then the tube was centrifuged for 5 minutes. Proper mixing of solution was ensured by vortex mixing. Supernatant of albumins and globulins were removed from mix using a micropipette. 70% (v/v) ethanol aqueous solution was prepared and 0.4 ml added per 100 mg of initial wheat flour to pellet in Eppendorf tube. The tube was swapped off in 5 minutes of pulsing and 5 minutes of vortex, for 30 minutes. Then the tube was centrifuged for 5 minutes. The supernatant of gliadins were removed from the mix using a micropipette. SDS phosphate buffer solution was then made and 0.5% (w/v) of SDS was added into a 0.05 M PBS solution. This solution was placed on a hot plate with a magnetic stirrer until it was completely homogeneous. Using the PBS and SDS solution, a 0.4 mL mixture was added per 100 mg of initial wheat flour to pellet in Eppendorf tube. The tube swapped off in 5 minutes of pulsing and 5 minutes of vortex, for 30 minutes. Then the tube was centrifuged for 5 minutes. Supernatant of SDS soluble glutenin subunits were removed from mix using a micropipette. After all the separation steps the resulting pellet consisted of insoluble SDS glutenin subunits which were shown to form amyloid fibrils. 1 M HCl at pH 1.6 was then added to the remaining pellet. The solution was pulsated for 5 minutes until homogeneity, and then placed inside the incubator at 55 °C for 72 hours, until signs of fibrillation.

Chart 1: Flowchart of Amyloid Fibril Formation from Wheat Flour



Different methods are being used for detecting amyloid fibrils, including attaching Thioflavin T (ThT) and employing fluorescence-based imaging; or by examination of the morphology by means of transmission electron microscopy (TEM); or confirmation of β-sheet secondary structure via Fourier transform infrared (FTIR) spectroscopy [5].

ThT binding and Confocal Microscopy: Thioflavin T is a benzothiozole dye with a high affinity for proteins containing β-sheet content and is popularly adopted "gold standard" for specific staining and identification of amyloid fibrils *ex vivo*, *in vitro*, and in animal model studies [5]. Unbound ThT dye has fluorescence excitation (from 385 nm to 450 nm), which on binding to β-sheet structure undergoes a characteristic spectral shift resulting in enhanced fluorescence emission (from 445 nm to 482 nm). Change in spectral shift is utilized for bifurcation of bound ThT and unbound ThT and consequently the presence of amyloid structures [6]. A concentration of 3.14 mM Thioflavin T was made with distilled water. A volume ratio of 1:2 was used for fibril solution to ThT solutions, as the two solutions were mixed together. 25 µL of the protein and ThT solutions were taken from each sample and placed onto a glass slide. A cover slip was placed on top of the solution and edges were sealed with nail paint. The slides were then evaluated under an Olympus Filter FV1000 Confocal Microscope.

Transmission Electron Microscopy (TEM): In order to confirm the presence of Amyloid fibrils and to study their morphology TEM analysis was performed. The sample preparation method described in reference [5] was followed to avoid artifacts in TEM images. TEM grids with formvar carbon coating 200 copper mesh was used and negative staining of protein sample was performed to produce contrast. First, 10 uL of Amyloid sample was propped on carbon coated side of TEM grid and excessive solution was wicked off by placing a Kimwipe at the edge of the grid. 10 uL of 2 % Uranyl Acetate (UA) solution was dropped on a TEM grid containing sample and kept on grid for 3 minutes. After 3 minutes, the excessive UA solution was removed on Kim wipes and the sample was prepared for TEM analysis. The FEI Tecnai G2 30 TEM (located at CEMAS) was used for the Transmission electron imaging, operating at 300 kV.

Principal Findings and Results:

Amyloid fibrils were first observed by 19th century's prominent German physician Rudolph Virchow in 1854 [7]. Ever since the proposed model by Pauling and Corey [8], most accepted structure is cross-B core having β-strands embedded in β-sheet and parallel to fiber axis where β-sheets lie perpendicular to the major axis of fiber [9]. Amyloid fibrils can be obtained by stable unfolding of functionally folded peptides as well as proteins [10]. Figure 1. represents stages involved in amyloid fibril formation under suitable physio-chemical conditions. Amyloid fibril formation occurs from intermediate unfolded peptides and proteins structure into stacking of β-strands [10].

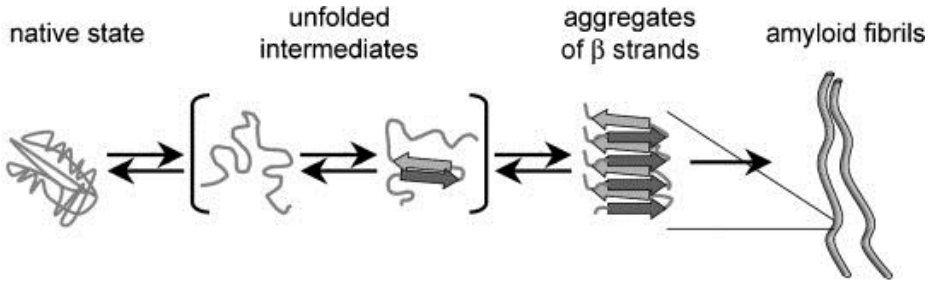


Figure 1 Stages involved in amyloid fibril formation [10].

Physical property measurements of amyloid fibrils revealed that their strength is as good as steel and silk fiber [11]. Moreover, Amyloid fibril's high Young's modulus is attributed to a dense network of hydrogen bonding, which leads to immense interaction between backbones of polypeptides [12,13,14]. Wetzel et. al. [15] summarized crucial similarities between amyloids, synthetic polymers and plastics: i) the assembly properties of amyloid and it's polymer subunits do not change under major chemical modifications; ii) Comparable isomorphism can be obtained from various monomeric units; iii) A condensed state forms via noncovalent interaction for both instances; iv) under specific conditions, gel or liquid crystals can form.

Wheat flour contains different proteins that can be classified as Albumins, Globulins, Gliadins and Glutenins [16]. Using Osborne solubility rules [17], each group of proteins was separated out. The only proteins to form amyloid fibrils under acidic conditions were SDS- insoluble subunits of the

Glutenins, the aim of the separations was to isolate those subunits. By attaching Thioflavin T (ThT) to these fibrils detection of their distribution within the CA mats was possible by means of fluorescence microscopy; The β-sheet secondary structure was discriminated via Fourier transform infrared (FTIR) spectroscopy, in order to confirm the presence of Amide I group. [5].

Presence of Amyloid fibrils: Due to the selective binding of ThT dye to amyloid fibrils, fluorescence imaging can be used to confirm their presence. Figure 2 represents images taken for samples prepared at different magnifications.

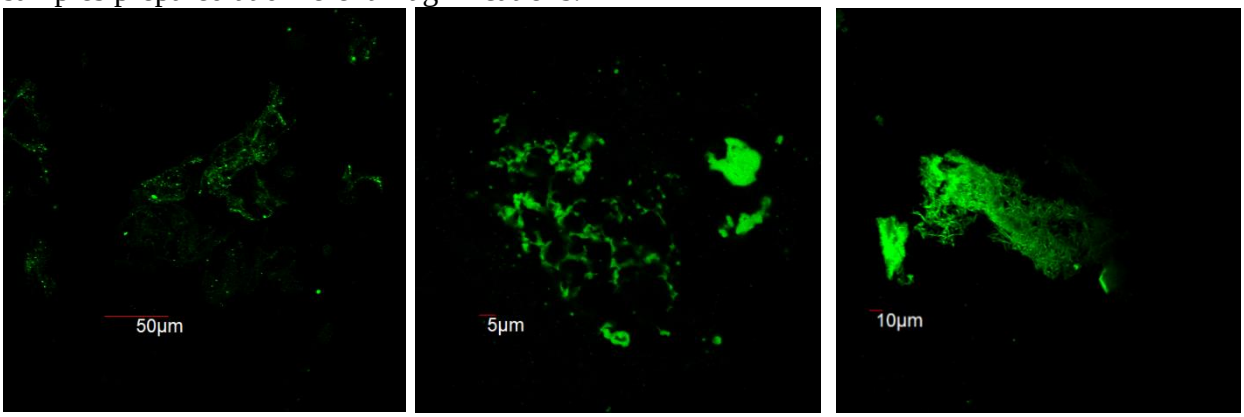


Figure 2 Confocal Microscopy image with fluorescence

Negative staining obtained from Uranyl Acetate produced contrast that made amyloid fibrils to appear dark in a bright background. Figure 3 represents images taken at different magnification. It is evident that uniformly dispersed amyloid fibrils are present with diameter ranging from 7-10 nm and length of 100 nm.

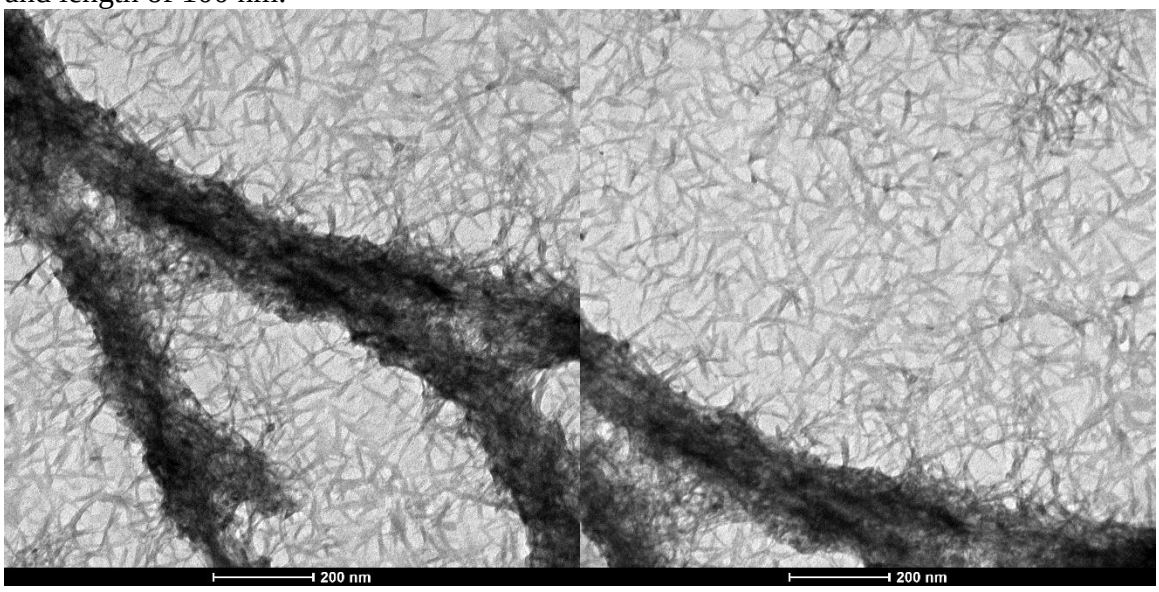


Figure 3 Transmission Electron Microscopy images of Amyloid fibrils

Finding Significance:

*Inexpensive and Scalable Synthesis of Amyloid Fibers as water filtration agents***:** In the previous research of our research group, hydrophobic (134° contact angle) and highly oleophilic membranes

were developed by the process of high-throughput electrospinning developed by the PI (Gouma) [18]. These filtration membranes consist of cellulose acetate and they are shown to be superior oil absorbents than any of the commercially available sorbents ones (see Figure 4). The mats were able to float on water for as long as 5 months and were able to remove petroleum products (even sheen oil) from the surface of the water.

Cellulose Acetate mats that exhibit water contact angle of 154.3° (super-water-repellant) were synthesized recently by our group[19]. Cellulose Acetate (CA) mat was prepared by electrospinning 15 wt % Cellulose Acetate Solution in 3:2 volume ratio of Acetone to Acetic acid. Solution was sonicated for 1 hour before electrospinning. 22-gauge needle, 20 kV of voltage, 7 cm of working distance and 1.2 ml/min flow rate was used as electrospinning parameters. Total of 10 ml 15 wt% CA solution was electrospun to make filter.

Preliminary studies explored the as-synthesized super hydrophobic cellulose acetate mats as a filtering membrane for fracking water. The results that are depicted in figure 4, shows that the single filtering step using these Nano fibrous mats transforms the fracking water to crystal clear water.

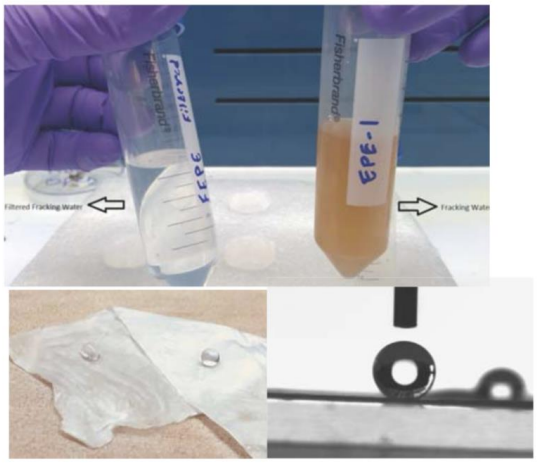


Figure 4: Fracking water after passing through electrospun nano-fiber CA filter

Water Purification Procedure: Fracking water was first passed through syringe filter of PVDF, 0.22 µm pore size in order to capture solid sediments. Once solid sediments were removed, this water sample was used for further treatment by the **Amyloid fibrils**. Vacuum purification was employed for fracking water using 0.22 µm pore size membranes. An electrospun Cellulose Acetate (CA) mat was place on the membrane of vacuum purification funnel and solution containing Amyloid fibrils (4 gm of Amyloid fibrils) was poured on top of CA mat. Top view of filter membrane with CA mat and Amyloid is presented in figure 5 below. 160 ml of fracking water was placed in top container of vacuum purification system and tube was attached to vacuum line to pass water through the membrane.

Semiquantitative ICP-MS analysis was run on fracking water and purified water by CA-Amyloid membrane. Concentration for different elements present in fracking water and purified water is presented in table 1 in appendix.

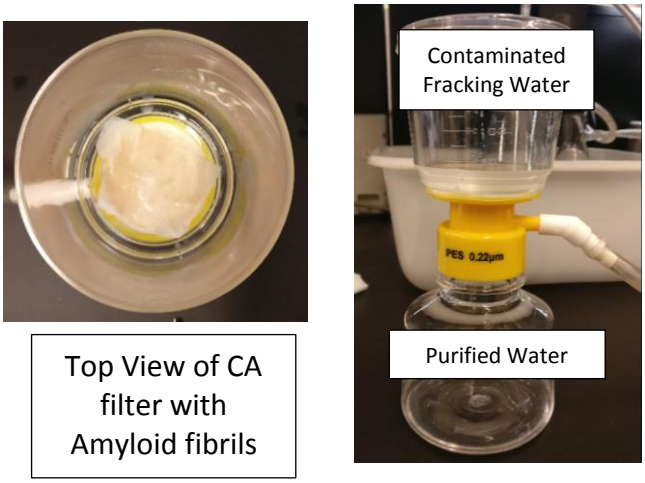


Figure 5: Left: top view of CA-Amyloid membrane Right: Vacuum filter

Acknowledgement: Dr. Shaurya Prakash's laboratory provided contaminated fracking water. Protein incubation and Water purification via Amyloid fibrils experiments were carried out in Dr. Harpreet Singh's laboratory from Molecular, Cellular and Developmental Biology Department.

References:

1. Bolisetty, S., & Mezzenga, R. (2016). Amyloid–carbon hybrid membranes for universal water purification. Nature nanotechnology, 11(4), 365.
2. Barrau, S., Zhang, F., Herland, A., Mammo, W., Andersson, M. R., & Inganäs, O. (2008). Integration of amyloid nanowires in organic solar cells. Applied Physics Letters, 93(2), 253.
3. Goesaert, H., Brijs, K., Veraverbeke, W. S., Courtin, C. M., Gebruers, K., & Delcour, J. A. (2005). Wheat flour constituents: how they impact bread quality, and how to impact their functionality. TIFS Trends in Food Science & Technology, 16(1), 12-30.
4. Osborne, T. B. (1907). The proteins of the wheat kernel. Washington, D.C.: Carnegie Institution.
5. Nilsson, M. R. (2004). Techniques to study amyloid fibril formation in vitro. Methods, 34(1), 151-160.
6. Biancalana, M., & Koide, S. (2010). Molecular mechanism of Thioflavin-T binding to amyloid fibrils. Biochimica et Biophysica Acta (BBA)-Proteins and Proteomics, 1804(7), 1405-1412.
7. Schultz, M. (2008). Rudolf Virchow. Emerging infectious diseases, 14(9), 1480.

8. Pauling, L., & Corey, R. B. (1951). The pleated sheet, a new layer configuration of polypeptide chains. Proceedings of the National Academy of Sciences, 37(5), 251-256.
9. Gras, S. L. (2007). Amyloid fibrils: from disease to design. New biomaterial applications for self-assembling cross-β fibrils. Australian journal of chemistry, 60(5), 333-342.
10. Cherny, I., & Gazit, E. (2008). Amyloids: not only pathological agents but also ordered nanomaterials. Angewandte Chemie International Edition, 47(22), 4062-4069.
11. Smith, J. F., Knowles, T. P., Dobson, C. M., MacPhee, C. E., & Welland, M. E. (2006). Characterization of the nanoscale properties of individual amyloid fibrils. Proceedings of the National Academy of Sciences, 103(43), 15806-15811.
12. Knowles, T. P., Fitzpatrick, A. W., Meehan, S., Mott, H. R., Vendruscolo, M., Dobson, C. M., & Welland, M. E. (2007). Role of intermolecular forces in defining material properties of protein nanofibrils. Science, 318(5858), 1900-1903.
13. Knowles, T. P., & Buehler, M. J. (2011). Nanomechanics of functional and pathological amyloid materials. Nature nanotechnology, 6(8), 469.
14. Adamcik, J., Lara, C., Usov, I., Jeong, J. S., Ruggeri, F. S., Dietler, G., ... & Mezzenga, R. (2012). Measurement of intrinsic properties of amyloid fibrils by the peak force QNM method. Nanoscale, 4(15), 4426-4429.
15. Wetzel, R., Shivaprasad, S., & Williams, A. D. (2007). Plasticity of amyloid fibrils. Biochemistry, 46(1), 1-10.
16. Goesaert, H., Brijs, K., Veraverbeke, W. S., Courtin, C. M., Gebruers, K., & Delcour, J. A. (2005). Wheat flour constituents: how they impact bread quality, and how to impact their functionality. TIFS Trends in Food Science & Technology, 16(1), 12-30.
17. Osborne, T. B. (1907). The proteins of the wheat kernel. Washington, D.C.: Carnegie Institution.
18. Sood, S., Divya, S., & Gouma, P. (2014). High throughput electrospinning of 3D nano fibrous mats. Journal of Nanoengineering and Nanomanufacturing, 4(1), 39-44.
19. Mikaeili, F., & Gouma, P. I. (2018). Super Water-Repellent Cellulose Acetate Mats. Scientific reports, 8(1), 12472.

Appendix:

Table 1: Elemental concentration in Fracking water and purified water

Element	filtered 10x (ppm)	Produced Fracking water 10x (ppm)
Cl	89,620	91,516
Ca	7,435	7,523
Sr	3,048	3,080
Br	2,547	2,579
Mg	1,229	1,245
Ba	539	541
K	342	345
Na	269	261
I	58	82
Li	44	45
Ti	14	14
Mn	4.1	4.2
Rb	1.9	1.8
Fe	1.0	0.7
B	1.0	0.9
Cs	0.93	0.97
Si	0.83	0.80
As	0.48	0.41
V	0.28	0.24
Zn	0.24	0.20
P	0.13	0.00
Rh	0.06	0.06
Eu	0.05	0.05
Ni	0.05	0.04
Cu	0.05	0.04
Tl	0.03	0.01
Al	0.02	0.01
Y	0.01	0.01
Co	0.01	0.01
In	0.01	0.00
Sb	0.01	0.01
Pb	0.01	0.00

2. Publication Citations

Materials Science & Technology 2018 | Columbus, OH
October 15th, 2018

3. Students Supported

This project supported three students from The Ohio State University. Reid Souchereau is a third-year undergraduate student majoring in Biomedical Engineering. Milind Pawar is a second year PhD student majoring in materials science and engineering. Owen Abe is a fourth year PhD candidate majoring in materials science and engineering.

4. Profession Placement of Graduates

At this time, none of the students who have been supported by this project has matriculated out of the university.

5.Awards or Achievements

An invention disclosure titled “Amyloid Fibers from Wheat Flour” was submitted at The Ohio State University in June of 2018.

Invention Disclosure	Received / Accepted	"Amyloid Fibers from Wheat Flour"	IDF-042138	6/29/2018	7/5/2018	7/9/2018

6.Any additional funding for this project

Several proposals have been submitted seeking funding for this work.