Proceedings of the 36th Biochemical Engineering Symposium

Larry E. Erickson
Kansas State University

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36th Biochemical Engineering Symposium
Kansas State University

Larry E. Erickson
Editor

April 21, 2007

Department of Chemical Engineering
Kansas State University
Manhattan, Kansas 66506
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FOREWORD

This work contains the Proceedings of the 36th Biochemical Engineering Symposium, which was held at Kansas State University on Saturday, April 21, 2007. The original schedule for the meeting is shown with all of the submitted presentations. Unfortunately, because of an auto accident, the students from Colorado State University were not able to arrive and make their presentations.

The actual program included 13 oral presentations and 9 posters. Some of the CSU manuscripts are included in the proceedings.

LIST OF PARTICIPANTS

Colorado State University - Dr. Ranil Wickramasinghe.

Iowa State University - Dr. Peter Reilly, Dr. Chuck Glatz, Alex Aguilar, Matthew Aspelund, Kerry Campbell, Ying Liu, Blake Mertz, Ramon Morales, Scott Munhall, Luis Petersen, Taran Shilling, Christopher Warner, and Bingqi Zhang.

South Dakota School of Mines and Technology - Dr. Patrick Gilcrease, Isha Chhatwal, Terran J. Elliott, Vo. D. Khang, and Geetha L. Muppidi.

University of Applied Sciences Giessen - Dr. Peter Czermak, Alexander Brix and Ronald Michalsky.


Kansas State University - Dr. Larry Erickson, Dr. L.T. Fan, Dr. Lorena Passarelli, Dr. Peter Pfommm, Dr. Mary Rezac, Dr. Praveen Vadlani, Andres Argoti, Chundi Cao, Juan Cruz, Deborah Hemphill, Jwan Ibbini, Jagadeswara Karra, Sathish Kumar Santharam, Tyler Selbe, Devinder Singh, Lynette Vera, Clinton Whiteley, Dr. Huicheng Xie, and Xiangxin Yang.
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Zehra Tosun, Thomas Crouzier, and Peter S. McFetridge, "Conducting Hydrogels/SWNT Composite for Nerve Tissue Engineering Applications," OU, Poster

Clinton Whiteley, Ryan Green, Terrie Boguski, and Larry E. Erickson, "Hazardous Materials Planning Tool," KSU, Poster

Bingqi Zhang, Ankit Agarwal, and Surya Mallapragada, "Injectable Self-Assembling Cationic Polymers for Gene Delivery," ISU, Poster
Tangential Flow Filtration of Aedes Aegypti Densonucleosis Virus

David L. Grzenia*, S. Ranil Wickramasinghe

Department of Chemical and Biological Engineering, Colorado State University,
Fort Collins, CO 80523-1370, USA

Abstract

Tangential flow filtration of virus particles may be an important unit operation for the purification of virus particles and virus vectors for gene therapy and viral vaccines. Here passage of 18-26 nm Aedes aegypti densonucleosis virus through 100 kDa membranes has been investigated. Consequently it is challenging to remove larger host cell proteins and DNA from the virus particles using membrane filtration.

Tangential flow ultrafiltration experiments have been conducted using two modes of operation. One mode, the high performed tangential flow filtration includes recycling of some of the permeate co current to the feed solution. The other mode uses a single pump for the feed while the permeate outlet is at atmospheric pressure. Conclusions were that the high performance tangential flow mode of operation gives better passage of virus particle. Further the permeate flux is more stable over longer periods of time.

Introduction

Gene therapy represents a modern molecular biology approach to medical treatment. Gene therapy-based methods treat diseases (e.g., cancer, arteriosclerosis, osteoporosis) by delivering DNA specifically to affected cells to re-instate cellular production of therapeutic proteins or peptides. Modified virus vectors represent one possible delivery vehicle. Similarly vaccine vector delivery systems are essential if vaccines are to be developed against viruses such as human immunodeficiency virus (HIV) (2). Promising delivery vectors for HIV vaccines include plasmid DNA and replication incompetent adenovirus (3-4). Large-scale purification of these delivery vectors will be essential if vaccines that are developed using methods of molecular biology are to be of clinical value.

Tangential flow microfiltration and ultrafiltration are frequently used in the biopharmaceutical industry for bioreactor harvesting, purification and concentration of protein products. While tangential flow filtration could be used for purification and concentration of virus particles few studies are available in the literature on the use of tangential flow filtration for the recovery and purification of virus particles (5). Virus particles are usually produced by a cell culture based system (6). Since virus particles vary in size from 20 to over 300 nm removal of large proteins and DNA from small virus particles using membrane filtration will be challenging.

* Corresponding author. Tel: 970-491-5252; Fax: 970-491-7369; Email: davidgrzenia@web.de.
We have investigated the use of tangential flow ultrafiltration for the purification of the parvovirus *Aedes aegypti* densonucleosis virus (AeDNV). The 18-26 nm wide AeDNV virus particle is highly pathogenic to *Aedes aegypti* which is a vector of the viruses that cause dengue and yellow fever. AeDNV could serve as a biological control agent with important applications in integrated vector control programs against mosquito-borne diseases. Advantages are set in easy handling of the virus particles, which are easy to grow using a cell culture based system. Consequently AeDNV could serve as a model for studying tangential flow filtration of parvoviruses.

In tangential flow ultrafiltration the feed is pumped on one side of the membrane while permeate is withdrawn from the other side. The permeate side of the membrane is exposed to atmospheric pressure. Since the feed side pressure decreases from the feed inlet to the feed outlet, the transmembrane pressure also decreases with increasing distance from the feed inlet. Van Reis et al (9) investigated the possibility of fractionation of proteins using tangential flow ultrafiltration. He describes a high performance tangential flow filtration (HPTF) mode of operation where some of the permeate is pumped parallel and co-current to the feed solution such that the feed solution is subjected to an approximately constant transmembrane pressure.

Here we have conducted tangential flow ultrafiltration of AeDNV using 100 kDa flat sheet membranes using both modes of operation. The effect on protein and virus passage through the membrane for the two different modes of operation has been investigated.

**Experimental**

**Production of AeDNV particles in serum free medium**

AeDNV particles were produced using the *A. albopictus* cell line C6/36 in a serum and protein free medium (SFPFM) (SF-900 II SFM, Invitrogen Corporation, Grand Island, NY). The C6/36 cell line was grown at 28 °C in T-75 flasks containing 14 ml SFPFM medium supplemented with 1% penicillin-streptomycin (Invitrogen Co., Carlsbad, CA). The pH of the medium was 7.4. When the cells reached 40-80% confluency, the *A. albopictus* cell line C6/36 was transfected with pUCA, an infectious clone containing the AeDNV genome, by using Qiagen effectine kit (Qiagen, Valencia, CA) (10). The media was changed 8 to 18 hours post transfection to remove the toxic Qiagen transfection solution. Four days post transfection, as a clear cell monolayer was visible, transfected C6/36 cells were then transferred from the T-75 flask to a 125 mL spin flask (stirred bioreactor) (Wheaton Science Products, Millville, NJ) at a cell concentration of 5.5×10^6 cell/mL. The total medium volume was 100 mL. The bioreactor was stirred at 300 rpm at 28 °C. Five to six days later, when the cell concentration reached around 5-6×10^6 cells/mL, cells were transferred to a second 500 mL bioreactor at a cell concentration of 5.5×10^6 cell/mL and a medium volume of 300 mL. About a week later, the cells were collected, frozen and thawed three times then centrifuged at 3,750 rpm for 15 min at 4 °C to remove cellular debris. The supernatant containing AeDNV particles was filtered using a 0.45 μm filter and stored at -80 °C for future use.
Tangential flow filtration

Experiments were conducted using flat sheet Sartocon® Slice 200 cassette (Sartorius AG, Germany). The Sartocon® Slice 200 cassette is 15 cm in length and has a molecular weight cut off (MWCO) of 100 kDa (Sartorius polyethersulfone 308 1466802E SG). The filtration area is of 200 cm² (0.02 m²). Figure 1 shows the experimental setup for the HPTF mode, when some of the permeate is pumped co current to the feed. Figure 2 shows the experimental setup when only a feed pump is used.

Figure 1: HPTF mode of operation

Figure 2: Single pump mode of operation
Prior to conducting virus filtration experiments DI water fluxes were measured at the operating conditions using the single pump mode of operation. Next HPTF filtration experiments were conducted. The feed for the HPTF mode of operation consisted of pure filtered supernatant containing AeDNV particles. The total volume was 500 ml. The feed was concentrated about 10 times resulting in a retentate volume of 50 ml. The retentate from the HPTF mode of operation was then diluted with SF-900 to a total volume of 500 ml. This solution became the feed for the single pump mode of operation.

In all the experiments, feed flow rate was set to 150 mL min\(^{-1}\) using a peristaltic pump. In the HPTF mode, feed was pumped to the membrane and retentate was returned to the feed reservoir. The permeate was measured by a balance (Mettler Toledo, Columbus, OH) and recorded by an online personal computer. Permeate, from the permeate reservoir, was pumped with a second peristaltic pump to the permeate inlet of the membrane module. The permeate flow rate was set to 10 mL min\(^{-1}\) (see Figure 1).

At the commencement of an experiment, the feed pump was started and the permeate outlet closed. For the single pump mode of operation after a 10-15 minutes the permeate outlet was opened. For the HPTF mode of operation after 10-15 minutes of operation the permeate outlet was opened and the co flow pump started. For both methods of operation, the mass of permeate collected was measured by a balance and recorded by an online personal computer.

For both modes of operation, the feed, retentate, permeate inlet and outlet pressures were measured by four micro switch sensing and control sensors (Honeywell International Inc., Morristown, NJ). The data were automatically recorded on a personal computer. The transmembrane pressure was calculated, using the following equation for the HPTF mode of operation.

\[
\text{TMP} = \frac{P_{\text{Feed}} + P_{\text{Retentate}}}{2} - \frac{P_{\text{Permeate inlet}} + P_{\text{Permeate outlet}}}{2}
\]

For the single pump mode of operation the permeate flux was calculated as:

\[
\text{TMP} = \frac{P_{\text{Feed}} + P_{\text{Retentate}}}{2} - \frac{P_{\text{Permeate}}}{2}
\]

The average transmembrane pressure for the HPTF mode of operation was around 0.155 bar with a variation from 0.138-0.172 bar. The average transmembrane pressure for the single pump mode of operation was around 0.372 bar with a variation from 0.379-0.345 bar. It is not surprising that the permeate pressure is higher for the HPTF mode of operation as the permeate line is open to the atmosphere. Some variation in transmembrane pressure, for both modes of operation, was caused by the action of the peristaltic pump. Pressure readings were accurate to ± 5% of the average value.

In both modes of operation, 1 mL samples were taken at regular intervals, from the feed, retentate and permeate for analysis of virus titer and protein concentration. This process was continued until 400-450 mL permeate had been collected i.e. the contents of the feed reservoir were concentrated about 10 times.

At the end of the virus filtration experiment, the membrane was flushed using DI water followed by 1 mol/L NaOH solution at 50 °C for 1 hour. Then the DI water flux was measured. After
cleaning it was ensured that the water flux was at least 95% of the initial water flux. The membrane was then stored in a 0.1 mol/L NaOH solution supplemented with 20% ethanol by volume.

Samples of the feed, retentate and permeate were analyzed for virus titer and protein concentration. All samples were analyzed in triplicate and average results reported.

**PCR assay**

A quantitative real time PCR (QPCR) based assay was used to determine the virus titer in the infective solutions as AeDNV does not show cytopathic effects. The primers and probe were designed within a conserved region of the viral NS1 gene. Primer Express® oligo design software (Applied Biosystems, Foster City, CA) was used to design forward primer: CAT ACT ACA CAT TCG TCC TCC ACA A, reverse primer: CTT GCT GAT TCT GGT TCT GAC TCT T, and TaqMan Probe: FAMCCA GGG CCA AGC AAG CGC CTAMRA. The reaction was performed in 96-well format skirted v-bottomed polypropylene microplates (MJ Research, Inc., Waltham, MA) with optical caps (Applied Biosystems, Foster City, CA).

The Brilliant® Quantitative polymerase chain reaction core reagent kit (Stratagene, La Jolla, CA) was used as the QPCR master mix. Each well consisted of 4 μL of unknown sample or standard control DNA pUCA plasmid, 10 μL master mix, 2 μL of 0.05 mmol/L forward primer, 2 μL of 0.05 mmol/L reverse primer, and 2 μL of 5x10⁻³ mmol/L probe. The thermal cycling conditions were: stage 1 50 °C for 2 min, stage 2 95 °C for 10 min, stage 3 95 °C for 15 sec, Stage 4 60 °C for 1 min and then stages 3 and 4 repeated 39 times. All reactions were performed in the Opticon 2 DNA Engine (MJ Research, Inc.). All samples were analyzed three times and average results are reported. The accuracy of the PCR assay was determined by analyzing 12 samples of the same infective solution and found to be within ± 0.5 log units. Further details of the PCR assay are given by Afanasiev et al 1999 (11).

**Protein assay**

Protein concentration was measured using a BCA Protein Assay Kit (Pierce, Rockford, IL) following the manufacturer’s instruction. Using a 96 well microplate (Nalge Nunc International) 25 μL of unknown sample or standard albumin were added to the wells. Next 200 μL of working reagent were added to each well. The plate was covered and incubated at 37 °C for 30 min. After cooling to room temperature, the absorbance of each sample at 562 nm was measured using a microplate spectrophotometer (Benchmark Plus Microplate Spectrophotometer, Bio-Rad Laboratories, Hercules, CA). As described by the manufacturer, the protein concentration is determined and reported with reference to a standard albumin solution provided by the manufacturer. All samples were analyzed in triplicate and average values reported. The accuracy of the protein assay is better than 6% of the mean. Since the working range of the assay is 20-2,000 mg/mL, samples were diluted 10 fold.
Results and Discussion

Figure 3 shows the variation of the relative permeate flux with permeate volume for 100 kDa membrane using the HPTF and single pump modes of operation. The relative permeate flux is determined by dividing the actual permeate flux by the pure water flux at the same operating conditions. The initial pure water flux was 130 Lm⁻²h⁻¹.

As can be seen the permeate flux for the HPTF mode of operation is lower yet more stable than the single pump mode of operation. The lower permeate flux for the HPTF mode of operation is not surprising as the average transmembrane pressure is lower. In addition some of the permeate is recycled through the permeate channel. As can be seen however, by ensuring a nearly constant transmembrane pressure as a function of distance along the module, the permeate flux is much more stable. Further, since the feed for the single pump mode of operation, is the diluted retentate from the HPTF mode of operation, we would expect the viscosity of the feed for the single pump mode of operation to be a little lower than the feed for the HPTF mode of operation due to removal of protein during the HPTF step. We would expect the feed to the HPTF step to be more fouling due to the greater protein concentration present in this feed. However it can be seen that the permeate flux for the HPTF mode of operation is more stable.

Figure 3: Variation of the relative permeate flux with permeate volume for serum free medium in HPTF and single pump mode of operation. The relative flux is defined as the measured flux divided by the water flux.

Figures 4 and 5 give the variation of the virus titer in the feed, retentate and permeate for the HPTF and single pump modes of operation. The solid line gives the measured virus titer for an experiment that contains growth medium but no virus particles. Thus to calculate the absolute virus titer, one must subtract the value given by the solid line from the titer measured for the infective solution.

Figures 4 and 5 indicate that the virus titer in the feed and retentate are similar. This is not unexpected as the permeate flow rate is much lower than the feed flow rate thus there is little virus concentrate in a single pass through the module. Figure 4 and 5 also indicate that for both
modes of operation, the virus titer in the feed and retentate is higher than the titer in the permeate. Consequently there is partial rejection of the virus by the membrane (4-5).

Comparing Figures 4 and 5 it can be seen that the virus titer for HPTF is much more stable than for the single pump mode of operation. Further the virus titer in the permeate for HPTF is closer to the feed titer when compared to the permeate and feed titers for the single pump mode of operation. Consequently by maintaining a constant transmembrane pressure drop along the length of the module, not only is the permeate flux more stable but passage of virus particles is also enhanced. These results consistent with the result obtained by van Reis et al (9) for protein filtration using HPTF.

Figures 6 and 7 give the protein concentration in the feed retentate and permeate for HPTF and single pump modes of operation. Since the medium is protein free, the protein assay is detecting the presence of polypeptides and amino acids, which are referred to for consistency as ‘proteins’. As can be seen the protein concentration for all three feed streams is similar for the HPTF mode of operation indicating little rejection of proteins.

Figure 4: Variation of the virus titer, in feed, retentate and permeate with permeate volume for serum free medium in HPTF mode of operation.
Figure 5: Variation of the virus titer in the feed, retentate and permeate with permeate volume for serum free medium in single pump mode of operation.

The results obtained here indicate that more stable permeate fluxes and better passage of virus particles is obtained for the HPTF mode of operation. While the results are consistent with those of van Reis (9) for protein filtration they suggest that using a lower molecular weight cut off membrane may result in passage host cell proteins and DNA but retention of virus particles. Consequently the HPTF mode of operation may be more suitable for purification of small virus particles where the virus particles are similar in size to some of the contaminant proteins and DNA.

Figure 6: Variation of protein concentration in the feed, retentate and permeate with permeate volume for serum free medium for HPTF mode of operation.
Figure 7: Variation of protein concentration in the feed, retentate and permeate with permeate volume for serum free medium for the single pump mode of operation

Conclusions

Tangential flow ultrafiltration of AeDNV has been conducted using HPTF and single pump modes of operation. Fact is, more stable permeate fluxes are obtained in the HPTF mode of operation. Further, better passage of virus particles is also observed for the HPTF mode of operation. Since separation of small virus particles from larger host cell proteins and DNA is of commercial interest, our results indicate that the HPTF mode of operation may be more suitable for separation of species where the size difference is less than an order of magnitude. The result obtained for 100 kDa membranes indicate that a lower molecular weight cut off membrane e.g. 50 kDa may reject virus particle but allow passage of larger proteins and DNA when run in the HPTF mode of operation.

Acknowledgements

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References


Kinetics and Mechanisms of Protease Assisted Aqueous Extraction of Soybean Oil

Kerry A. Campbell¹, Ramón C. Morales¹, Tracey M. Pepper², Charles E. Glatz¹

¹Department of Chemical and Biological Engineering and ²Bessey Microscopy Facility, Iowa State University, Ames, IA 50011

Abstract

The objectives of this research were to identify mechanisms of oil release and determine optimal conditions for free oil yield for enzyme-assisted aqueous extraction of soybean oil as a basis for rational process design and optimization. Light microscopy (LM) and transmission electron microscopy (TEM) were used to study the effects of physical treatments on cellular disruption, to identify extraction barriers, and to deduce important extraction parameters. Selected extraction parameters were optimized using Response Surface Design. Parameters included solid-liquid ratio, enzyme/substrate ratio and agitation rate. All parameters tested had significant effects for both extraction yield and demulsification yield, although solid-liquid ratio had by far the largest effect. The demulsification step had the most influence on determining optimal parameter values for overall free oil yield. Kinetic experiments showed that the extraction could be modeled as a two-pool model with first order kinetics.

Introduction

Because of the low bulk density of bio-based feedstocks, the transition from a petroleum-based to a bio-based economy will require the development of small-scale distributed production centers rather than large centralized facilities seen in industry today (Brown, 2003). In the case of vegetable oil production, the necessity of large extraction plants is created in part by the high capital cost associated with handling hazardous organic solvents, which are vital for attaining high extraction yields. To improve the economics of small scale vegetable oil production, it is necessary to find a cost-effective alternative to hazardous organic solvent processes.

Aqueous extraction processing (AEP) is one such alternative. Aqueous processing of soy is nothing new as water-based methods of recovering soy protein have existed for more than 2,000 years (Hagenmaier, 1997). In more recent decades, processes for the aqueous extraction of oil were developed at Texas A&M University (Johnson and Lusas, 1983). However, low oil yields from AEP have prevented commercialization of this process. Aqueous extraction is further complicated by the fact that the oil is recovered as a very stable emulsion, referred to in this paper as cream. The enzyme-assisted aqueous extraction process (EAEP) increases yields (Yoon et al. 1991) and has the potential to reduce emulsion stability allowing the recovery of free oil.

Four basic steps in EAEP are 1) particle size reduction of the soy by grinding, extrusion, flaking, or a combination of these; 2) extraction in water with enzyme treatment; 3) centrifugal separation of the solid, aqueous (milk), and cream (oil) phases; and 4) demulsification to recover free oil (Lusas, E. W. and Jividen, H. M., 1987). In order to develop a process design, it will be necessary to understand how extraction parameters affect total oil yield. That is, investigators
must not only study how parameters affect oil extraction yield, but they must also consider how easily the resulting emulsion can be broken.

In contrast with solvent extraction, where oil itself is extracted from the solid phase, AEP extracts water-soluble material from the solid phase, allowing the release of oil (Rosenthal et al., 1998). Therefore, dissolution of these "barriers" is vital for oil removal. Microscopy shows that the majority of the intracellular material of soy cotyledons is protein bodies and oil bodies (Bair, 1979). It follows that the important parameters are those which affect the accessibility of these cellular contents to the extraction medium, and those which affect the stability and solubility of oil and protein bodies. These parameters are pH, particle size, solid-liquid ratio, enzyme concentration, agitation rate, extraction time, and temperature (Rosenthal et al., 2001, Lusas et al., 1982). Agitation rate and solid-liquid ratio are also important parameters affecting emulsion stability (Rosenthal 1996, Embong et al., 1977). There have not been any published studies that optimized soy oil extraction with demulsification, something that is necessary for a process design, nor has microscopy been used to confirm the extraction mechanisms deduced by experimental research. The objectives of this research are to study the mechanisms of oil release using microscopy, determine optimal operating conditions for free oil yield, and characterize extraction kinetics for rational process design.

Materials and Methods

Materials

Full-fat soybean flour was prepared by the Center for Crops Utilization and Research at Iowa State University from a 2005 soybean crop harvested locally. Flour was stored at -20°C until use. Proteases Protex 7L and Protex 6L were provided by Genencor International, Inc., Rochester, NY.

Extraction

Extractions were performed in a 2-L jacketed glass reaction vessel (Model 4742, Chemglass Inc., Vineland, NJ) held at 50°C by a circulating water bath and agitated by a stirrer (Model BDC 3030, Caframo, Ltd., Wiarton, Ontario) with a 1-inch, 3-bladed screw impeller. Constant pH of 8.0 was maintained using an autotitrator (Model 718 Stat Titrino, Metrohm, Ltd., Herisau, Switzerland). The extraction was carried out in two steps. First, material was extracted in DI water without enzyme with an agitation rate of 500 rpm for 120 min. Then, enzyme was added and agitation rate was changed to the test condition for an additional 120 min. At the end of the extraction, two 35-mL samples were withdrawn by siphon from the center of the reactor. Samples were centrifuged at 3,000 x g for 15 min. Sample fractions (solid and liquid) were massed, and solid fractions were retained for analysis. Solid fraction moisture content was determined by drying in an oven at 130°C for 12–15 h. Solid fraction oil content was determined on freeze-dried solid samples in a Goldfisch apparatus. Solid and oil yields were determined by taking the difference between content of the starting material and the content of the solid phase. The remaining extraction material was centrifuged to remove solids, and the liquid phase was placed overnight in a separatory funnel kept at 4°C. After 12–15 h in the funnel, the milk phase was drained off and discarded, while the cream was retained for demulsification.
Demulsification

Five grams of soybean cream were mixed with 1 g of distilled water in a 250-mL beaker and bacterial neutral endoprotease Protex 6L (Genencor) was added at a concentration of 1% w/w. The samples were shaken in an incubator shaker (Model C24, New Brunswick Scientific, Edison, NJ) at 120 rpm for 3 h and 50°C. The enzyme-treated cream was transferred to a test tube (17 mm internal diameter and 100 mm height) and enzyme deactivation was performed by heating the samples in a water bath at 95°C for 5 min. The cream was then centrifuged again at the same conditions reported above. The free oil released after centrifugation was removed with a plastic pipette and weighted for oil quantification. Demulsification yield was calculated as the mass of free oil recovered divided by the total oil of the cream. Because of the small quantities of cream recovered, individual samples could not be characterized for total oil content. Therefore, all cream samples were assumed to be the same composition as a non-enzyme-assisted aqueous-extracted cream extracted under similar conditions, previously determined to be 55% oil.

Microscopy

For transmission electron microscopy (TEM), samples were prepared following procedures by Bair (1979). Dried material was imbibed overnight in deionized water. Wet material was placed directly in fixative solution. Tissues were fixed in 2% glutaraldehyde (w/v) and 2% paraformaldehyde (w/v) in 0.1 M cacodylate buffer, pH 7.2, for 48 h at 4°C. Samples were rinsed 2 times in 0.1 M cacodylate buffer, pH 7.2, and then fixed in 1% osmium tetroxide in 0.1 M cacodylate buffer for 1 h at room temperature. The samples were then dehydrated in a graded ethanol series, cleared with ultra-pure acetone, infiltrated and embedded using a spurr’s epoxy resin (Electron Microscopy Sciences, Ft. Washington, PA). Resin blocks were polymerized for 48 h at 65°C. Thick and ultrathin sections were made using a Reichert Ultracut S ultramicrotome (Leeds Precision Instruments, Minneapolis, MN). Thick sections were contrast-stained using 1% toluidine blue. Ultrathin sections were collected onto copper grids and counter-stained with 5% aqueous uranyl acetate for 15 min. followed by Sato’s lead stain for 10 min. Images were captured using a JEOL 1200EX scanning and transmission electron microscope (Japan Electron Optic Laboratories, Peabody, MA). Light microscopy (LM) images were captured using a Zeiss Axioplan 2 light microscope (Carl Zeiss MicroImaging, Inc., Thornwood, NY).

Experimental Design and Statistical Analysis

A Response Surface Experimental Design similar to one used by Rosenthal et al. (2001) was used to test factor effects and to develop an empirical model for process optimization. A Box-Behnkin design with three center points was selected, with three factors: solid-liquid ratio (S), enzyme-solid ratio (E/S) and agitation rate (A). Following Rosenthal et al., S was coded linearly, while E and A were both coded exponentially according to the following equations:

\[ A = \ln\left(\frac{R_A}{500}\right) \]  \hspace{1cm} (1)

\[ E = 1.4 \ln(C_e) \]  \hspace{1cm} (2)

\[ S = \frac{R_S-L}{0.05} \]  \hspace{1cm} (3)
where $R_A$ is the agitation rate, $C_e$ is the enzyme concentration (w/dry wt), and $R_{s-L}$ is the solid-liquid ratio. The levels were chosen to be spaced evenly either linearly or exponentially (Table 1). Responses measured were oil extraction yield, defined as the fraction of oil in the starting material remaining in the solid phase, and demulsification yield, defined as the fraction of oil in the emulsion recovered as free oil. Statistical analysis was completed using JMP 6.0 statistical software package by SAS, Inc., Cary, NC.

Table 1 - Factor levels chosen for surface response design experiment.

<table>
<thead>
<tr>
<th>Level</th>
<th>S</th>
<th>E/S</th>
<th>A</th>
</tr>
</thead>
<tbody>
<tr>
<td>-</td>
<td>0.05</td>
<td>0.5%</td>
<td>200</td>
</tr>
<tr>
<td>0</td>
<td>0.10</td>
<td>1.0%</td>
<td>500</td>
</tr>
<tr>
<td>+</td>
<td>0.15</td>
<td>2.0%</td>
<td>1500</td>
</tr>
</tbody>
</table>

Results and Discussion

Microscopy

Typical cotyledon cells are about 30 μm in diameter and 70–80 μm long (Bair, 1979). Figure 1 shows a TEM of a native soybean cotyledon radial cross-section. The most significant component of the soybean cotyledon is protein bodies, where about 80% of the soy protein is stored. The oil is stored in small organelles called oil bodies, which fill most of the cytoplasmic network (Bair, 1979).

LM and TEM images of the material at different stages of extraction are shown in Figure 2. The starting material contains some clumps of intact cells, but much of the material seems to exist as free cellular material from disrupted cells. In the images of extracted material, no free cellular material appears outside of cells. In comparing the two different extraction treatments, there is no noticeable effect of enzyme treatment. Some cells appear to have been partially extracted with coalesced oil droplets formed inside, others appear completely empty, and others appear completely untouched by the extraction. Presumably, extraction of the soluble material destabilizes the oil bodies and allows them to coalesce into large droplets. However, cellular disruption must be sufficient that droplets and other less soluble material can be released into the extraction medium. From the completely unextracted cells, it can be assumed that the cell wall barrier was completely intact, preventing any of the solutes from escaping from the cell.

These findings may have several implications in the extraction parameters. First, it can be stated that cellular disruption is a prerequisite for protein and oil extraction. Second, to maximize extraction from the disrupted cells, anything that can be done to aid in the removal of the cellular contents will increase yield. Two ways to accomplish this can be by increasing the solubility of the cellular contents, i.e. with a proteolytic enzyme, or by further physical action on the flour, i.e. shearing by agitation. This is the justification for choosing the extraction parameters for the response surface model design and optimization.

Response Surface Design Experiment

Analysis of variance of the response surface data shows that all of the linear and quadratic effect terms are significant to a 0.10 α-level, with no significant interaction effects. The fact that all the parameters tested had significant effects supports the hypothesis built around the extraction mechanism deduced from microscopy above. Keeping only the significant terms gives par-
ameter values shown in Table 2, with the resulting model shown in Equation 4. To test the fit of this model, predicted values are plotted against experimental values, and are overlaid a line with a slope of unity (Figure). The fit appears to be very good, with an R-squared value of 0.99. The data also appear randomly distributed about a line with a slope of unity, another indication of a good model fit.

Figure 1. TEM cross section of a soybean cotyledon. PB = protein body; OB = oil body; CW = cell wall; N = cell nucleus.

Figure 2. TEM and LM of starting material, solid material after 2 h of aqueous extraction, and solid material after an additional 2 h of enzyme-assisted aqueous extraction. a = starting material;
b = AEP extracted material; c = EAEP extracted material; 1 = LM; 2 = TEM; FM = free cellular material; PB = protein body; CO = coalesced oil; UE = unextracted cell, PE = partially extracted cell.

The demulsification yield showed similar results. All parameters had significant linear and quadratic effects to the 0.10 level except agitation, for which the linear term was not significant. The resulting model is shown in Equation 5. The model fit was not as good as for the extraction yield but is still acceptable, with an R-squared value of 0.93, and points randomly distributed about a line with a slope of unity.

Table 2. Parameters and relative sizes of significant effects for oil extraction yield (a) and demulsification yield (b).

\[
Y_{\text{extraction}} = 0.8001 - 0.052S + 0.0063E/S - 0.0068E/S^2 + 0.0049A + 0.0084A^2
\]

\[
Y_{\text{demulsification}} = 74.9 + 24.9S - 25.3S^2 + 18.7E/S - 13.6E/S^2 - 2.3A - 22.6A^2
\]
Experimental repeatability of demulsification was also not as good as for extraction, as indicated by the large prediction bands. However, the yield ranges covered are very large, going from zero to near 100%. Therefore, even with poor prediction bands, this model is still adequate for qualitative predictions of emulsion stability, i.e. high, medium, or low demulsification yield.

Analyzing the size of the parameter effects can be useful in making deductions about the extraction mechanisms. For extraction yield, the size of the parameter effects from agitation is roughly equal to the effect of the enzyme, illustrating that both of these factors are of equal importance in enhancing extraction yield. Still, the factor that has the greatest influence on determining oil extraction yield is the solid-liquid ratio, which has a parameter estimate value an order of magnitude larger than any of the other extraction yield terms. For demulsification, on the other hand, all significant parameters are of the same magnitude. Based on this, it can be said that enzyme concentration and agitation rate are important more because of their effect on emulsion stability rather than from their marginal enhancement they provide for extraction yield.

This is illustrated when using these two models to determine an overall optimal operating condition. Assuming all the extracted oil goes into the cream, free oil yield is then the product of extraction yield and demulsification yield. Finding the maximum of this combined model using a trial-and-error algorithm from the Solver add-in for Microsoft Excel (2003 version) gives parameter values and yields shown in Table 3, with a maximum possible free oil yield of 68%. These parameter values are the same that arise when maximizing demulsification yield alone, not taking extraction yield into account at all. So it appears extraction yield must be sacrificed to achieve a cream than can be more completely demulsified. It should also be noted that even if the two models accurately predict yields, 68% may not be the actual yield because a significant amount of extracted oil may be present in the aqueous phase. Overall material balances covering all phases of the extract have yet to be completed.

Table 3. Conditions and yield for overall optimization of free oil recovery.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Coded value</th>
<th>Actual value</th>
<th>Units</th>
</tr>
</thead>
<tbody>
<tr>
<td>S</td>
<td>0.40</td>
<td>0.12</td>
<td>Solid/water</td>
</tr>
<tr>
<td>E/S</td>
<td>0.68</td>
<td>1.60</td>
<td>wt/wt</td>
</tr>
<tr>
<td>A</td>
<td>-0.04</td>
<td>474</td>
<td>RPM</td>
</tr>
<tr>
<td>$Y_{\text{extraction}}$</td>
<td>78.2%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$Y_{\text{demulsification}}$</td>
<td>87%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$Y_{\text{free oil}}$</td>
<td>68%</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Extraction Kinetics**

In order to develop a process design, the extraction kinetics must also be determined. A preliminary experiment examined this at conditions close to the optimal condition discussed above ($S = 0.10$, $A = 500$ rpm) but in the absence of enzyme, with samples drawn every 20 min
and analyzed for extraction yield. A two-pool model with first-order kinetics of the form of Equation 6 was fit to the data using nonlinear regression techniques.

\[ Y_e = P_o + P_1 \left( 1 - \exp(-k_1 t) \right) \]

where \( Y_e \) = extraction yield
\( P_o \) = static extraction pool, i.e. the fraction extracted immediately upon mixing
\( P_1 \) = time-dependent extraction pool
\( k_1 \) = first order rate constant (\( \text{min}^{-1} \))
\( t \) = time (min)

The extraction results of two trials and parameter values are shown in Figure 3 and Table 4. The repeatability of two trials indicates this method works well for measuring extraction kinetics in the absence of enzyme. Based on the microscopy results, the \( P_o \) extraction pool could be interpreted to be the material from cells that have a very high degree of disruption, and is therefore extracted virtually immediately upon mixing with water, and the \( P_1 \) pool would be the material extracted from cells of a lower degree of disruption.

Table 4. Parameter values for model fit to yield kinetic data for extraction without enzyme.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Solids Yield</th>
<th>Oil Yield</th>
</tr>
</thead>
<tbody>
<tr>
<td>( P_o )</td>
<td>0.62</td>
<td>0.62</td>
</tr>
<tr>
<td>( P_1 )</td>
<td>0.07</td>
<td>0.13</td>
</tr>
<tr>
<td>( k_1 )</td>
<td>0.057</td>
<td>0.045</td>
</tr>
</tbody>
</table>

Figure 3. Solids and oil extraction yield kinetics without enzyme. Extraction conditions were \( S = 0.10, A = 500 \) rpm.
Conclusions and Follow-up

In order to develop a process design for an enzyme-assisted aqueous extraction process for soy, the extraction mechanism and kinetics must be adequately understood. This involves understanding which extraction parameters are important and how these affect extraction yield and emulsion stability.

Microscopy of flour from soy flakes before and after extraction indicated that cells must be disrupted in order for extraction to occur. Also, additional physical treatments may enhance the extraction from cells with a low degree of disruption. A Response Surface Design experiment showed that proteolytic enzymes and increased agitation rate did indeed increase extraction yield, but that the effect of these factors was small compared to the effect of solid-liquid ratio. Furthermore, demulsification is the most important step in determining free oil yield, and overall optimization of this process shows that extraction yield must be sacrificed in order to achieve an emulsion that can be broken.

Kinetic experiments showed that the extraction can be modeled as a two-pool first-order kinetic model. One extraction pool enters solution immediately upon mixing, presumably from cells of a high degree of disruption, with a slower extraction pool presumably from cells of a low degree of disruption.

The empirical models developed here must still be validated before an optimal extraction condition can be established. Once this is done, the kinetics can be characterized and the overall material balance can be established to be used for a process design and economic analysis.

Acknowledgments

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Destabilization of Emulsion Formed During Aqueous Extraction of Soybean Oil

Ramon Morales, Hyun-Jung Kim, Cheng Zhang, Charles E. Glatz, Stephanie Jung

Abstract Characterization and destabilization of the emulsion formed during aqueous extraction of oil from soy flour were investigated. This emulsion was collected as a cream layer and was subjected to various single and combined treatments, including thermal treatments and enzymatic treatments, aimed at recovery of free oil. Whereas heat treatment alone did not modify the free oil recovery, freeze-thaw treatment increased the oil yield from 3% to 22%. After enzymatic treatment of the emulsion, the oil recovery increased to 23% while the particle size of the enzyme-treated soy emulsion changed from 5 μm to 14 μm. The peptide profile of the proteins located at the emulsion interface was significantly modified after enzymatic treatment; the molecular weight of the enzyme-treated proteins was much smaller. This result suggested that smaller peptides were less able to stabilize the emulsion. When enzymatic treatment was followed by a freezing-thawing step, the oil recovery increased to 46%. The particle size of the emulsion that underwent this treatment increased to 25 μm, indicating coalescence among oil droplets. This result can be attributed to the thinner interfacial membrane after enzymatic hydrolysis, partial coalescence during freeze-thaw, and coalescence after centrifugation.

Introduction

Soybeans are a major crop in the United States, grown for vegetable oil and protein. Defatted soybean meal is largely used for feeding livestock (97%), however an increasingly variety of food and industrial uses have been developed [1,2]. Hexane extraction is currently the most cost-effective oil recovery method for oilseed processing, leading to a meal with a residual oil content below 1% [3]. But hexane is highly flammable, toxic, and possibly carcinogenic, and ends up as fugitive emissions of volatile organic compounds (VOCs). These safety, environmental, and health concerns have generated interest for replacement of hazardous organic solvent extraction [4]. Aqueous extraction processing (AEP) replaces hexane with water as a promising alternative for soybean oil extraction that enables the simultaneous production of oil and protein using an environmentally clean technology with potential value-added products without the hazards of hexane [5]. However, when water is used to extract oil from soy material the release of oil is lower and only a small proportion of the oil is released as free oil, whereas the majority is emulsified, mainly in a cream layer but also in the aqueous phase (skim milk).

Emulsion stability depends on surfactant properties such as hydrophobicity, conformation, solubility, the competition with other surfactants, and the system pH and concentration [6]. These properties affect creaming, coalescence, and flocculation of the emulsion and may be manipulated for destabilization. For this reason, emulsion stability in food products has been extensively studied, and processing parameters able to modify this stability have also been identified. Enzymatic, physical, and physicochemical treatments all reduce emulsion stability [7]. The majority of studies have been performed on model emulsions that control both concentration
and composition. Few studies have investigated naturally-occurring emulsion stability and, to our knowledge, no studies have reported means to break naturally-occurring soybean emulsions formed during aqueous extraction. In the soybean, triacylglycerol (TAG) is at the core of oil bodies stabilized by an outer layer of phospholipids (PLs) and basic proteins, termed oleosins [8]. Upon extraction the oil bodies may be disrupted but also further stabilized by coextracted proteins such as the major soy storage proteins glycinin and β-conglycinin.

In this study the approach was two-fold. First, the composition of the cream formed during aqueous extraction of soybean flour was determined in order to identify the likely emulsifying agents. Second, enzymatic and thermal treatments applied alone and in combination, were evaluated for their effectiveness in destabilizing this emulsion, thereby achieving a higher yield of free oil.

Experimental Procedures

Materials

Soybean flour was prepared in the pilot plant of the Center for Crops Utilization Research at Iowa State University and stored at 4°C. The flour was prepared from variety IA 1008 soybeans. The soybeans were cracked, the hulls were aspirated, and the meats were milled twice with a pin mill. The oil content of the flour, determined by the Goldfisch method [9], was 22% and the crude protein content, determined by the Dumas method [9] with a conversion factor of 6.25, was 48.5% on a dry basis.

Oil Extraction and Phase Separation

Extractions were conducted in a 2-L reaction vessel (Model CG-1926-03, ChemGlass Inc., Vineland, NJ). The flour (200 g) was dispersed at 200 rpm in 2 L of distilled water at 50°C. The pH of the dispersion was adjusted to 8 with 2 N NaOH. After pH stabilization, stirring continued for 15 min. After extraction, the sample was left for 1 h on a laboratory bench to cool at 25°C. Sodium azide (0.04%) was added to the cooled sample to prevent microbial growth. Insoluble fraction, aqueous phase, and cream were separated by centrifugation in a 250-mL centrifuge bottles at 3,000 x g and 25°C for 15 min (Sorvall RC-5B, Newtown, CT) using a HS-4 swinging bucket rotor. After centrifugation, the cream layer (oil emulsion) was located at the top of the supernatant. The cream was collected on top of a 200-mesh sieve by decanting the supernatant gently through the screen.

Enzymatic Treatment

Thirteen grams of soybean cream were mixed with 1.45 g of distilled water in a 250-mL beaker. The pH was adjusted to 8 and Protex 7L (bacterial neutral endoprotease kindly provided by Genencor International Inc, Rochester, NY) was added at 1% w/w of cream. The samples were shaken in an incubator shaker (Model C24, New Brunswick Scientific, Edison, NJ) at 115 rpm and 50°C for 3 h. The enzyme-treated cream was transferred to 30-mL glass centrifuge test tubes and the enzyme was deactivated by heating in a water bath at 95°C for 5 min. The tubes were then centrifuged at 3,000 x g for 15 min and 25°C. The free oil released after centrifugation was removed with a plastic pipette and weighed for oil quantification.
Thermal Treatment
The enzyme-treated cream and corresponding control (cream treated as above but without Protex 7L addition) were heated at 95°C for an additional 30-min period after enzyme deactivation, and cooled in a chilled water bath before centrifugation was performed at the same conditions previously given. For the freeze-thaw treatment, the enzyme-treated cream and corresponding control were held in a freezer (Model Isotemp, Fisher Scientific, Pittsburgh, PA) at -18°C for 24 h. The samples were then thawed by incubating at 30°C for 3 h before centrifugation.

Particle Size Distribution
The volume-weighted mean diameters ($D_{4,3}$) of the emulsion obtained after treatments and before centrifugation were measured by laser light scattering particle size analyzer (Mastersizer 2000 S, Malvern Instruments, Ltd., Chicago, IL). Small portions of the sample were dispersed in 50 mL of distilled water by vortexing for 30 s before the analysis was performed. The refractive index (RI) used for the soybean oil droplets was 1.47 and 1.333 for the dispersant [10]. The absorption value was set up at 0.001. All measurements were carried out at 25°C.

Cream Characterization
The cream, before and after treatment, was prepared for characterization using the method of Hunt et al. [12] and Agboola et al. [13, 14]. A flowchart of the detailed procedure for cream characterization is shown in Fig. 1. The cream was washed by dispersion into 4 parts of distilled water then recovered by centrifugation (15,000 x g for 30 min at 25°C; Sorvall RC-5B centrifuge, Newtown, CT) using a fixed angle rotor (Model SLC-1500, Kendro, Ashville, NC) and decanting onto a 200-mesh sieve. The washed cream was then stored at 4°C before analysis; protein content of the cream was from nitrogen content determination (Rapid NIII combustion analyzer; Elementar Americas, Inc., Mt. Laurel, NJ) using a factor of 6.25 to convert the percentage of nitrogen to crude protein content. Total carbohydrate content was determined using the method of Fox et al. [11]. The solids content was determined gravimetrically after drying at 105°C (Stabil-Therm oven; Blue M Electric Company, Blue Island, IL) with measurements taken over 24 h until constant mass was reached. Crude oil, TAG and PL, were isolated from the cream by the Folch method [15], which includes an extraction step with a chloroform/methanol mixture (2:1 v/v) followed by a wash step with a methanol/water mixture (1:1 v/v). The collected oil extract was evaporated under vacuum in a rotary evaporator for 30 min at 25°C to remove chloroform, methanol, and trace amounts of water. TAG and PL were fractionated by a counter-current extraction method [16]. The TAG was evaporated in a fume hood to remove hexane; the difference between the crude oil and the TAG was considered as the PL yield.

Proteins of the washed cream were isolated by acetone precipitation [17] where ice-cold acetone was added to the cream in a ratio of 20:1 (v/w). The solution was mixed, incubated at -18°C for 2 h, and then centrifuged at 14,000 x g for 15 min at 4°C. The supernatant was removed and the precipitate was washed 4 to 5 times with cold acetone until no yellow color was seen in the solvent. The protein pellet was air dried at 25°C to remove residual acetone.

The dried protein pellet (approximately 5 mg) was then dispersed in 1 mL of a solution of 2% SDS, 8 M urea and 50 mM DTT. Protein samples were combined with an equal volume of sample buffer containing 200 mM Tris–HCl pH 6.8, 2% SDS, 40% glycerol, 0.04% Coomassie Blue G-250 and 350 mM of DTT and heated at 100°C for 5 min before being loaded to a Ready Gel Tris–HCl, 4–15% resolving gel (Bio-Rad, Cat # 161-1104, Hercules, CA) and a Ready Gel
Tris–tricine, 16.5% resolving gel (BioRad, Cat # 161-1107, Hercules, CA). SDS–PAGE was performed on these protein fractions and run at 200 V on a Mini-PROTEAN® II Electrophoresis Cell (Bio-Rad, Hercules, CA). The loading amount of protein into the gel was 10 μg. After electrophoresis, the gels were stained with Coomassie Brilliant Blue.

The protein molecular weight distribution was calculated from the densitometric measurement of protein bands on the gel with the software ImageJ [18]. The protein content and profile of the enzyme-treated cream were compared to those of the control.

The surface protein concentration, Γ, was calculated according to Agboola [13,14]:

\[
Γ = \frac{M_{p,o}}{SSA}
\]  

where \(M_{p,o}\), the mass ratio of protein to oil, was from the cream composition and SSA, the specific surface area of the oil droplets, was found to be 6.13 m²/g from the particle size distribution, \(D_{3,2}\), and the soybean oil density, 0.92 g/cm³, using the following equation [13,14]:

\[
SSA = \frac{6 \times 1}{D_{3,2} \times ρ_{oil}}
\]  

**Statistical Analysis**

A screening experimental design with five factors: enzyme treatment (E), heat treatment (H), freeze-thaw treatment (F-T), enzyme + heat treatment (E-H) and enzyme + freeze-thaw treatment (E-FT), was used and analyzed with JMP 6 (SAS Institute Inc., Cary, NC). The responses were the particle size (\(D_{4,3}\)) and the free oil recovery (%). All the measurements for oil recovery and particle size determination were done in triplicate and analyzed by the one-way analysis of variance statistical method (ANOVA). The means from the cream composition and from each treatment were compared by using the Tukey’s Honestly Significant Differences (HSD) test. The level of significance was defined as \(p \leq 0.05\).

**Results and Discussion**

**Characterization of Soybean Oil Emulsion**

The two major components of the cream (Table 1) formed during aqueous extraction processing, TAG and water, lesser quantities of proteins, carbohydrates, and PLs, are available for stabilization. Tzen et al. [8] reported that proteins and PLs stabilize the oil bodies in the soybean seed. The presence of both components in this cream could account for the emulsion formation after oil release by AEP. Carbohydrates might also play a role in the emulsion stability, but they do not work as emulsifier alone. Rather, carbohydrates could interact with proteins to form complex interfacial structures to enhance stability. Other parameters such as PLs probably play an important role in this emulsion stability as well [19].

Surface protein concentration is an index that can be used to characterize emulsion stability. Tcholakova et al. [20] has shown that a surface protein concentration of 1–2 mg/m² was the smallest coverage of oil droplet to form a monolayer to ensure a stable emulsion. The surface
protein concentration of our emulsion, calculated by means of Eq. 1 was 14.65 mg/m²; this value indicates a stable multilayer emulsion [20].

The densitometry analysis of the SDS–PAGE gel of the surface proteins from the untreated-washed cream (Fig. 2) revealed that about 40% of the proteins have a molecular weight (MW) higher than 30 kDa; also the subunits of the two distinctive soy storage proteins, glycinnin and β-conglycinin, were seen in the gel. Dickinson [19] reported that high MW protein emulsifiers form strong viscoelastic interfacial films between droplets and prevent coalescence, which is thought to be one of the major mechanisms underlying demulsification [7]. The stability of the soybean oil emulsion formed during AEP might, therefore, be due to the presence of multilayer protein emulsifiers of high MWs. Accordingly, the thermal and enzymatic treatments chosen in this study aimed the destabilization of these multilayer proteins. It has been reported that thermal treatments at high temperatures lead to the denaturation and aggregation of the interfacial proteins, promoting coalescence [7, 22]. Indeed the two major soy proteins, β-conglycinin and glycinnin, have denaturation temperatures around 75 °C and 93°C, respectively [21]. In addition, it is hypothesized that freeze and thaw promotes partial coalescence due to the high concentration of oil droplets within the frozen emulsion. Also, during freezing the lipid droplets come into close contact, thus favoring droplet-droplet interactions [23, 24]. Fullbrook [25] reported that reduction in the size of the proteins/peptides at the emulsion interface might enhance oil coalescence; therefore properties of the soybean cream, after hydrolysis of the surface proteins into small peptides using an endoprotease was also investigated.

Thermal Treatment
The heat and the freeze/thaw treatments increased the D₄₃ from 5 µm to 14 µm (Table 2). Centrifugation did not impact the free oil yield of the heat-treated cream, whereas an oil yield of 22% was obtained for the freeze/thaw-treated cream, compared to 3% of the control (Fig. 4). The particle size distribution of the untreated cream (control) was bimodal (Fig. 3) and it was slightly modified after thermal treatments (Fig. 3A). The particle size distribution profile for these two treatments showed broader peaks, indicating that important population of larger droplets was formed (Fig. 3A). Separation of the oil phase from the aqueous phase could be facilitated by centrifugation of the emulsion because centrifugal forces pushed the droplets to one end of the container, bringing them together to promote coalescence [7].

Protein Profile Analysis
The peptides present at the droplet interface were characterized by SDS–PAGE (Fig. 5). The subunits of the two major soy proteins, glycinnin and β-conglycinin, were seen in the gel for the untreated cream. After enzymatic treatment of the oil emulsion the corresponding bands for glycinnin and β-conglycinin, among others, disappeared and peptides of low MW were observed. This fact provided evidence that the oil emulsion was partially destabilized when small peptides were present at the interface of the oil droplets. This was also confirmed by the recovery of free oil after centrifugation.

Enzymatic Treatment
Enzymatic treatment by itself modified significantly the mean particle diameter of the soybean oil emulsion from 5 µm to 14 µm (Table 2). The particle size distribution profile of the cream treated at 1% w/w (Fig. 3A) shows a narrow peak in the 50–90 µm area. It was also noticed that the peak observed in the 0.1–1 µm area was less pronounced. It can be hypothesized
that after the enzymatic hydrolysis, the interfacial membrane became thinner and less rigid. This possible change in addition to both the incubation conditions (heating and continuous mechanical agitation through shaking) and the proximity of the oil droplets due to the high oil content may have promoted the increase of droplet diameter by collisions and coalescence [7]. Indeed, the peptide profile of the enzymatic treated cream was significantly modified compared to the control (Fig. 5). All the peptides from the enzyme-treated cream had a molecular weight lower than 14 kDa. This modification of the peptide profile at the emulsion interface did not promote coalescence before centrifugation but probably played a key role in the increase of oil yield to 23% after centrifugation (Fig. 4). It can therefore be concluded that the enzymatic treatment improved interactions between droplets and debilitated the emulsion interface, leading to release of free oil, probably due to coalescence after centrifugation.

**Combination of Enzymatic and Thermal Treatment**

When the cream was first subjected to enzymatic treatment followed by heat treatment, the mean particle diameter of the soybean oil emulsion increased from 14 µm to 27 µm (Table 2). The particle size distribution of the enzyme/heat-treated samples were comparable to the one obtained after enzymatic treatment alone but with significant decrease of the volume fraction of particles in the 0.5-1 µm range (Fig. 3B). Oil recovery was also enhanced since enzyme/heat treatment led to 29% oil recovery compared to 5% by heat treatment alone (Fig. 4).

Combination of enzymatic and freeze/thaw treatment also improved the mean droplet diameter of the emulsion, from 15 µm, corresponding to the freeze/thaw treatment, to 25 µm (Table 2). The particle size distribution profile showed that a broader peak was obtained from this combinatorial treatment compared to the enzyme/heat treatment, thus a larger population of flocs was formed (Fig. 3B). Also, enzyme/freeze/thaw treatment led to a higher free oil recovery of 46% compared to 22% of the freeze/thaw treatment with no enzyme addition (Fig. 4). From the increase in the mean particle size and oil recovery, it can be concluded that coalescence of the oil droplets were promoted when physical and enzymatic treatments were applied successively. These treatments increased the oil droplet size and reduce the surface protein concentration of the membrane making it thinner and partially destabilizing the emulsion. The peptide profile of the thermal-treated samples was similar to the one obtained for the control (untreated cream) whereas small peptides were observed in the cream on which enzymatic treatment, alone or in combination with thermal treatment, was applied (Fig. 5).

**Conclusion**

It can be concluded from the soybean oil emulsion characterization that the stability of the soybean oil emulsions is partially due to the presence of proteins of high molecular weights and the multilayer nature of the interface. Other parameters such as PLs could contribute to emulsion stability, but they were out of the scope of this study and further investigation would be necessary. Demulsification of the soybean cream was analyzed in the present study with enzyme and/or thermal action. Improvement of the free oil recovery yields were obtained with combination of freeze/thaw and enzymatic treatments. This combinatorial treatment also increased significantly the mean particle size of the oil droplets in the emulsion. This was thought by the thinner interfacial membrane due to the decrease in surface protein concentration after enzymatic hydrolysis, partial coalescence during freeze-thaw and coalescence after centrifugation. This promising result should contribute to the feasibility of AEP to obtain free oil.
Acknowledgments

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References


**Table 1.** Percentage composition of soybean oil emulsion*.

<table>
<thead>
<tr>
<th></th>
<th>Oil*</th>
<th>Water</th>
<th>Proteins</th>
<th>Carbohydrates</th>
<th>Phospholipids</th>
</tr>
</thead>
<tbody>
<tr>
<td>Soy cream</td>
<td>59 ± 5.7</td>
<td>35 ± 6.4</td>
<td>5 ± 0.69</td>
<td>1.3 ± 0.23</td>
<td>0.8 ± 0.14</td>
</tr>
</tbody>
</table>

*Mean ± 90% confidence intervals from triplicates.

* TAG content.

**Table 2.** Effect of treatments on the mean droplet diameter (D_{4,3}) of the soybean oil emulsion before centrifugation*.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>D_{4,3} [μm]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated cream (control)</td>
<td>5 ± 0.27^a</td>
</tr>
<tr>
<td>Heat</td>
<td>14 ± 0.48^b</td>
</tr>
<tr>
<td>Freeze/Thaw</td>
<td>15 ± 0.59^b</td>
</tr>
<tr>
<td>Enzymatic (1% wt)</td>
<td>14 ± 1.26^b</td>
</tr>
<tr>
<td>Enzyme/Heat</td>
<td>27 ± 3.00^c</td>
</tr>
<tr>
<td>Enzyme/Freeze/Thaw</td>
<td>25 ± 4.06^c</td>
</tr>
</tbody>
</table>

* Values represent mean ± 90% confidence intervals from triplicates. Values with different letters are significantly different at p < 0.05.
Soy Cream from AEP

Wash by centrifugation 15,000 x g for 30 min

Supernatant (Washed cream)

Crude protein content
Carbohydrate content
Moisture content

Acetone precipitation

Surface protein

Re-solubilization

Molecular weight distribution by SDS-PAGE

Extraction with chloroform/methanol

Folch wash

Crude oil content (TG & phospholipids)

Countercurrent separation

Phospholipids
Triacylglycerol

Fig. 1. Flow diagram for characterization of the oil emulsion obtained during AEP of full-fat soybean flour.

<table>
<thead>
<tr>
<th>kDa</th>
<th>1</th>
<th>2</th>
</tr>
</thead>
<tbody>
<tr>
<td>M.W. range</td>
<td>Mass percent (%)</td>
<td></td>
</tr>
<tr>
<td>&gt;55 kDa</td>
<td>11.6</td>
<td></td>
</tr>
<tr>
<td>30–55 kDa</td>
<td>27.8</td>
<td></td>
</tr>
<tr>
<td>&lt;30 kDa</td>
<td>60.7</td>
<td></td>
</tr>
</tbody>
</table>

Fig. 2. SDS–PAGE profile of the proteins in the washed cream. Lane 1, MW markers (10–100 kDa range); lane 2, cream proteins. The corresponding protein MW distribution based on densitometry is also shown.
Fig. 3. Particle size distribution profile for treated creams. C – control, E – enzymatic treatment, H – heat treatment; F–T – freeze/thaw treatment; E–H – enzymatic treatment followed by heat; E–FT – enzymatic followed by freeze/thaw.

Fig. 4. Effect of different treatments on oil recovery yields after destabilization of the emulsion.

Fig. 5. SDS-PAGE profile of soybean oil emulsion subjected to various treatments. Gel A: Ready Gel Tris–HCl, 4–15% resolving gel. Lane 1: MW marker (6.5–66 kDa range); 2: untreated cream, 3: enzymatic treatment, 4: heat treatment, 5: freeze/thaw treatment, 6: enzymatic treatment followed by heat treatment, 7: enzymatic treatment followed by freeze/thaw treatment. Gel B: Ready Gel Tris–tricine, 16.5% resolving gel. Lane 1: MW marker (3.5–26 kDa range); 2: untreated cream; 3: enzymatic treatment.
Development of highly active enzyme preparations for use in organic solvents based on fumed silica

Juan C. Cruz, Kerstin Würges**, Peter Czermak**, Peter Pfornm, Mary Rezac

Department of Chemical Engineering, Kansas State University, Manhattan, KS 66506-5102
**University of Applied Sciences Giessen, Giessen, Germany

Abstract

We are reporting on a new strategy for enzyme activation in organic solvents. We have successfully improved the catalytic activity of subtilisin Carlsberg (serine proteinase) in n-hexane through freeze-drying in the presence of fumed silica which consists of sintered silicon dioxide nanoparticles. The use of this enzyme preparation as a model for biotransformations in organic solvents has been explored both in batch and continuous packed bed experiments. We have hypothesized that these unexpected results are related to overcoming of mass transfer limitations by the using inert high specific surface area supports for the immobilization. We are planning to expand the application of our fumed silica activation procedure in solvents to other enzymes such as lipases for esterification and transesterification reactions, and organophosphorous hydrolases for destruction of chemical warfare agents and pesticides. We are also considering experiments to investigate immobilized enzymes on silica at the single-enzyme level.

# current address: Institut für Biotechnologie 2, Forschungszentrum Jülich, Jülich, Germany
Cloning, expression, and purification of a glycoside hydrolase family 44 cellulase from Clostridium acetobutylicum in Escherichia coli

Taran C. Shilling¹, Clark F. Ford², and Peter J. Reilly¹

Departments of ¹Chemical and Biological Engineering and ²Food Science and Human Nutrition, Iowa State University, Ames, IA 50011

Introduction

One of the challenges facing the transition to cellulosic-based ethanol is the development of better enzymes for the hydrolysis of cellulose to simpler sugars for fermentation to ethanol. This is the context for this research project, which aims to study a poorly understood glycoside hydrolase (GH) family of cellulases. Glycoside hydrolase family 44 (GH44) is composed of primarily endoglucanases that hydrolyze β-1,4-glycosidic bonds with an inverting mechanism (Coutinho and Henrissat, 1999a,b). Currently there is no published three-dimensional crystal structure for this family and the catalytic acid and base are unknown. Since family classification is based upon primary sequence homology, enzymes within a family will have similar structural features and mechanisms, so determining the structure of one member of this family would shed light on the active-site structures of all GH44 members.

Contained within the cellulosome of Clostridium acetobutylicum ATCC 824 is the gene CAC0915, which putatively encodes a single-catalytic domain GH44 protein with a C-terminal dockerin domain. This GH44 putative protein, identified as CAC0915, has 606 amino acids for a calculated molecular weight of 66.8 kDa (Nölling et al. 2001) with a domain structure consisting of a signal peptide, a GH44 catalytic domain, and a type I dockerin. Aside from identification by homology as a GH44 member, this protein has not yet been produced for cellulase characterization outside of this current research.

The goal of this research is to study that GH44 catalytic domain by synthesizing the gene, ligating it into an expression vector, and transforming it into Escherichia coli, then producing the recombinant protein by fermentation and purification through chromatography and other methods. After sufficiently pure recombinant protein is generated, we will attempt to crystallize it and then to subject it to X-ray diffraction. We will then analyze the diffraction pattern to determine the tertiary structure and thus generate novel information about GH44 members.

Cloning Experiments

Recombinant protein expression can be limited by many factors, including the difference in codon bias between the source organism and the expression organism. Different organisms have differences in codon usage, or preferred codons for translation to the same amino acids. Even with high-expression vectors, conflicts in codon usage between the source and and host organisms can pose challenges to successful protein expression (Makoff et al. 1989, Gustafsson et al. 2004). The codon usages for C. acetobutylicum ATCC 824 and E. coli K–12 W3110 were compared and showed some key differences between the two organisms. To sidestep the challenge of isolating CAC0915 from a C. acetobutylicum culture and to account for codon bias to increase the probability of expressing CAC0915, we decided to synthesize the cDNA from scratch.
The cDNA sequence for CAC0915 was obtained from the Entrez nucleotide database (www.ncbi.nlm.nih.gov/Entrez/) and analyzed using the web-based sequence alignment program Pfam (www.sanger.ac.uk/Software/Pfam/) to investigate the possible domain structure of the expressed protein. The bulk of the protein, PfamB, is preceded by a region identified as a signal peptide and transmembrane region and is followed by possibly two dockerin regions interspersed with low-complexity regions. These latter regions were not studied because they are notoriously difficult to crystallize, and the focus of this research is on the CelA protein, not on dockerins.

The software Protein2DNA (DNA 2.0, www.dnatwopointo.com) was used to adjust the codon bias of CAC0915 and to add necessary functionality for cloning the desired gene. The first 1643 nucleotides of CAC0915, corresponding to the signal peptide through PfamB, were altered to conform within tolerance to the codon usage frequency of E. coli, prevent commonly used restriction sites, and avoid potentially problematic secondary structural DNA sequences. This sequence was synthesized from scratch by Megabase (Lincoln, NE), using their patented technology, PCRJet®. The complete and accurate gene was blunt-end-ligated into the plasmid pST1Blue and transformed into the blue/white screening host E. coli XL1-Blue (Stratagene). Positive transformants were selected and sequenced, confirming that the sequence accuracy. Deposited glycerol stock as well as the extracted gene in pST1Blue (Invitrogen) plasmid were provided in both forms to our laboratory.

Later it became apparent that only the PfamB region should be produced for crystallization, and so the provided DNA was used as template material. New primers were prepared and the PfamB region was amplified by PCR and ligated into the pGEM®-T Easy vector and transformed into E. coli Novablue. Subsequently, the gene fragment designed for pET-22b+ (Novagen) and was transformed into E. coli BL21 (DE3). The PfamB fragment was also amplified with different primers and ligated into pGEM®-T Easy, but was not successfully ligated into pET-11a. The pET-22b+ vector attaches a C-terminus histidine tag onto the gene fragment while the pET-11a vector would have produced just the PfamB gene fragment. Note in fact that there are slight additions to the expressed sequences. Translation begins with the methionine codon which precedes the PfamB region for both systems. At the C-terminus, pET-11a ends with the last residue of the Pfam region but the C-His protein includes the translated XhoI restriction site plus the six histidine residues, resulting in the addition of LEHHHHHHH before ending translation at the stop codon in the pET-22b vector. These subsequent cloning experiments were laborious but involved well-established details and those are omitted from these proceedings. However, it should be noted that the gene fragments were sequenced in the pGEM®-T Easy vectors by the ISU DNA Facility and were shown to be accurate.

Protein Production and Purification

Initial fermentations were performed to identify suitable growth media, induction concentrations, and the time after induction before harvesting products. A 50-mL starter inoculum was prepared from the deposited C–His PfamB stock and grown overnight and used to inoculate four fermentation flasks to initial OD600 values of 0.2. These four 250-mL flasks consisted of 50 mL of LB medium with carbenicillin antibiotic pressure (LB/Carb medium) and were used to test the benefit of supplemental 1% glucose and IPTG induction concentrations of either 0.4 mM or 1 mM. The cultures were grown at 37°C and shaken at 250 rpm to an OD600 of approximately 0.5
before induction. Samples were collected from each flask immediately before and after induction and at 1, 2, 3, and 6 h after induction.

Expression of the C-His protein was determined by SDS–PAGE analysis using a 4–20% polyacrylamide gradient gel (Bio–Rad) and stained with Coomassie Blue. There is a clear increase in the expression of the C-His protein for each of the four fermentation conditions after induction. Based on these results (not shown), the expression of the recombinant protein reaches a maximal level within 2 to 3 h after induction, and 0.4 mM IPTG is a sufficient induction concentration. There are no apparent differences to the expression caused by addition of 1% glucose to those media. Samples of the cellular material were also used for a qualitative assay of enzymatic activity. The cells were put through four freeze-thaw cycles between the -20°C freezer and the 37°C water bath and the lysed cell debris was spotted onto pH 7 plates with 0.5% CMC and 50 mM potassium phosphate. They were then incubated overnight before staining with 3 mg/mL Congo Red reagent and destaining with 1 M NaCl (Theather and Wood 1982). The cell lysates, created yellow clearing zones on the red-stained plates, clearly showing that the recombinant C-His protein has endoglucanase activity.

Lindwall et al. (2000) presented a methodology for screening a sparse matrix of buffer conditions to identify components favorable for solubilizing a recombinant protein. Twenty buffers were prepared and screened with cellular material from a new fermentation (LB/Carb media) following the screening protocol. These soluble fractions were stored at 4°C overnight and then visualized by SDS–PAGE. Only three buffers, 6, 18, and 28, solubilize the target protein well. Buffer 6 is 100 mM potassium phosphate, 50 mM (NH₄)₂SO₄, and 1% Triton X-100 at pH 6.0. Buffer 18 is 100 mM Tris, 100 mM KCl, 2mM EDTA, and 1% Triton X-100 at pH 8.2. Buffer 28 is 100 mM HEPES, 50 mM LiCl, and 0.1% deoxycholate at pH 7.0. Buffer 4, which was the worst buffer, is 100 mM HEPES and 100 mM KCl at pH 7.0 (Lindwall et al. 2000). The most and least effective buffers have some common components but their pHs vary.

Agarose plates were prepared for buffers 6, 18, and 28, with buffer 4 as a negative control, each with 0.5% CMC. Spots of the soluble fractions from buffers 4, 6, 18, and 28 were placed onto the buffer 28 CMC plate and incubated overnight at 37°C before Congo Red staining. The result was that only the soluble fraction in buffer 28 was active; it was therefore selected as an acceptable buffer for maintaining the solubility of the recombinant C-His protein after cell lysis.

The temperature stability of the PfamB-expressed protein was evaluated qualitatively using the Congo Red assay. Spots of 5 µL of the soluble fractions in buffers 4 and 28 were applied to agarose plates suspended in their own buffer and containing 0.5% CMC. Five plates for each pairing were used, each at a different temperature and spotted for different incubation times. This study revealed that 1 h or preferably 3 h is sufficient time for enzyme activity to be detected by the Congo Red method with this particular enzyme. Also, the temperature study appears to indicate that the optimal temperature range for activity of this enzyme is between 22 and 30°C, based on the intensity of the clearing zones. The activity is noticeably decreased at both 4°C and 37°C and almost negligible at 50°C. A final conclusion is that the enzyme appears to be stable for at least 24 h at 30°C, based on the observation that the 24-h incubation time resulted in a larger clearing zone than for 12 h, as was also apparent for the room temperature plate. Based on this observation, it was determined that the enzyme could be purified at room temperature without significant enzyme degradation.
A new fermentation of 500 mL of LB/Carb media was inoculated using a starter inoculum giving a very low OD$_{600}$, and then grown until an OD$_{600}$ of 0.57. At that point, IPTG was added to 0.4 mM and the culture was grown overnight before harvesting 12 h after induction. The cells and broth were separated by centrifugation at 10,000g for 15 min and the cell pellet was resuspended in 4 mL of buffer 28 followed by a second centrifugation step, after which the pellet was again resuspended in 4 mL of buffer 28. Lysozyme was added to the mixture, which was incubated on ice for 5 min and then sonicated intermittently at 35% power output for 5 min. After incubation on ice for 10 min, the soluble fraction was isolated by centrifugation at 16,000g for another 10 min. The soluble fraction was decanted from the cellular debris and stored at 4°C.

A 12-cm high, 0.8-cm diameter FPLC column containing Ni-NTA His-Bind$^\text{®}$ Superflow$^\text{TM}$ (Novagen) was prepared with 10 mL resin and operated at room temperature. The column was equilibrated with the recommended binding buffer (50 mM Tris-HCl, pH 8.0, 300 mM NaCl, 10 mM imidazole) and then 1 mL of soluble fraction was syringe-filtered (Costar 8110 uStar, 0.22 μm) and applied to the column through the pump. The flow rate was set at approximately 1 mL/min and this entire procedure follows Novagen's recommended operating protocol for FPLC purification of native proteins. The nonbinding proteins passed through the column within 1-2 column volumes, while binding buffer continued to pass through until the absorbance baselined. Approximately eight column volumes of wash buffer (50 mM Tris-HCl, pH 8.0, 300 mM NaCl, 20 mM imidazole) was used to further rinse the column. No proteins were released during the wash phase. The elution was performed with 50 mM Tris-HCl, pH 8.0, 300 mM NaCl, and 250 mM imidazole. A single peak was eluted at a volume approximately equal to the column volume. Multiple passes were run through the column (Figure 1). A second round of binding, loading, washing, and elution were essentially identical to the first round. Following the second round, the pooled nonbinding fractions from the first two runs were loaded onto the column. No proteins were released during this last elution step, indicating that the column had been adequately binding all of the possible protein.

![Figure 1](image)

Figure 1. Multiple purification runs of the fermentation soluble fraction on a Ni-NTA column. Peaks A1, A2, and C are nonbinding peaks, B1 and B2 are elution peaks, and the buffer stages are shown on the UV$_{280}$ chromatogram. Plot direction is right to left.

Key fermentation and purification fractions through this stage were analyzed by the previously described Congo Red procedure (Figure 2). Several observations should be made about the results of this qualitative test for enzymatic activity. The most important is that the elution fractions from the Ni-NTA affinity runs contain the recombinant protein and it is still active. The nonbinding fraction shows virtually no activity, which confirmed the chromatographic results,
which showed that this fraction contained no recombinant protein. A second major observation is that there had been noticeable losses of protein during the processing stages. The fermentation broth showed weak activity, but it was also quite dilute, and this could account for a significant amount of the protein. Since this protein is produced without a signal peptide, it will not be excreted by the cell, and so the activity seen likely results from prematurely lysed cells. Rinsing the cells to remove any contaminating proteins from the broth is probably not necessary and it resulted in protein loss. Also, the insoluble fraction contained activity that could likely be minimized if the insoluble fraction were resuspended in additional buffer and then centrifuged a second time.

Figure 2. Endoglucanase (CMCase) activity of the CelA fusion protein. 1) 20-μL nonbinding Ni–NTA fraction; 2) 20-μL cell pellet rinse in Lindwall buffer 28; 3) 20-μL pooled Ni–NTA elution peaks; 4) 5-μL soluble fraction; 5) 5-μL insoluble fraction debris; 6) 20-μL fermentation broth.

Bulk production fermentation and processing

Since the preliminary stages of purification were established by the work previously described, a large production run was performed. The stock of CFU C3, stored at -80°C and consisting of the CACFR11 insert ligated into pET-22b and transformed into E. coli BL21 (DE3), was streaked onto a LB/Carb agar plate. After growth for 10 h at 37°C, a single colony was selected and used to inoculate 200 mL of LB/Carb media. This culture was split into two 250-mL flasks and grown overnight at 37°C with 250-rpm shaking. The inoculum OD600 was 2.5 and the cells were harvested, resuspended, and used to inoculate the main fermentation, as described before. The main fermentation consisted of 3.3 L of LB/Carb media at an initial OD600 of 0.053, split between four cheesecloth-covered 2.8-L culture flasks, and grown at room temperature with shaking at 250 rpm. The growth of a single flask was monitored until the OD600 was greater than 1. At that point all of the flasks mixed and their combined OD600 was 1.49. A quarter of the culture was partitioned as a noninduced control, while the remainder was induced with 0.4 mM IPTG and divided between the remaining three flasks. The growth of noninduced and induced cultures was monitored hourly, with 1-mL samples being taken, centrifuged, decanted, boiled, and frozen as described before.

The fermentation growth curve and SDS–PAGE analysis of the protein production during the fermentation were performed (Figure 3). The fermentation was induced during the logarithmic growth phase and harvested while the culture was still growing. Induction with IPTG had a negligible effect on the growth rate compared to the noninduced control, and although this allowed the culture to continue to increase in cell mass, there is room to optimize the recombinant protein production. Still, the induced culture does produce the CelA fusion protein, as shown by SDS–PAGE analysis, and in greater amounts as the fermentation progressed after induction, shown...
with purified protein as a standard. The CelA fusion protein stained negatively, meaning that it was translucent with silver stain (Bio-Rad), and additional staining with Coomassie Blue was done.

Figure 3. (Left) OD<sub>600</sub> growth curves of noninduced and induced cultures of the CelA fusion protein. Induction occurred at 5.25 h followed by harvest after 4 h later. (Right) Completely solubilized fermentation protein samples of CelA fusion protein (marked) on a Bio-Rad Ready Gel® 4-20% polyacrylamide gel. 1) Broad-range standard; 2) purified protein; 3) preinduction; 4) induced +2 h; 5) induced +3 h; 6) induced +4 h (harvest); 7) noninduced control; 8) noninduced +2 h; 9) noninduced +3 h; 10) noninduced +4 h (harvest). Culture samples were normalized OD<sub>600</sub>.

The SDS-PAGE gel was handled in the following way: 40 µL of the boiled, preinduced culture sample was loaded, with the other samples normalized based on OD. Samples were diluted with 2x reducing buffer (125 mM Tris-HCl, pH 6.8, 20% glycerol, 10% 2-mercaptoethanol, 4% SDS, 0.0025% bromophenol blue), each to a 1x concentration except for the preinduced sample, which ended at 0.2x, limited by the initial sample volume and the gel well sizes. Standards and samples were boiled for 4 min, loaded into a 4-20% polyacrylamide gel (Bio-Rad) and run at 108 V with initial and final currents of 170 and 120 mA, respectively. The 52-min run was followed by silver staining (Bio-Rad) and Coomassie Blue staining.

The fermentation harvest followed a similar but slightly modified procedure to the previously described method. Both noninduced and the combined induced cultures were handled separately and all fermentation fractions were stored on ice until processing could be performed. Processing these fractions was laborious, taking 6 h and was performed immediately after the 9-h production fermentation.

The bulk of the cells and broth were separated by centrifugation at 10,000g and 4°C for 20 min. The masses of the cell pellets were 4.5 and 11.5 g for the noninduced and induced cultures, respectively. The cell pellets were resuspended in buffer 28 to final volumes of 6 and 21 mL, respectively. Lysozyme and RNAse A (both Sigma) were added to each mixture to final concentrations of 1 and 200 µg/mL, respectively. The cell suspensions were incubated on ice for at least
20 min before being sonicated at 30% power output for 4.5 min, on ice and with occasional swirling. Soluble fractions were collected after centrifugation at 16,000g in a prechilled 4°C rotor. The volumes of each soluble fraction collected were 1.5 and 12.5 mL for the noninduced and induced cultures, respectively.

The decanted cell broth of the induced cultures was ultrafiltered using a Model 8050 stirred cell (Amicon) with a 30-kDa MWCO (molecular weight cut-off) polyether sulfone (PES) membrane (Millipore). The broth was concentrated from approximately 2.4 L to 500 mL and the noninduced broth was not concentrated.

Samples of 15 mL of each culture, set aside before centrifugation, were processed by osmotic shock to release periplasmic proteins (Tkac et al. 2004). Cell pellets from the samples isolated were centrifuged at 6,000g for 10 min at 4°C and then were each resuspended in 250 μL of 20 mM Tris-HCl and 6 mM EDTA at pH 7.4. Samples were stored on ice for 45 min, followed by adding protease inhibitor and 250 μL of 40% w/v sucrose. The Sigma protease inhibitor cocktail, prepared following the manufacturer’s protocol, was used, with approximately 20 mg added per sample, which in retrospect was 10x greater than suggested. Following incubation at room temperature for 5 min, the samples were centrifuged at 14,000g for 10 min at 4°C and the soluble periplasmic fractions were collected.

Purification by chromatography through affinity to a Ni–NTA column

The concentration of the induced culture soluble fraction was measured by absorbance at 280/260 nm as 3.2 mg/mL, for a total protein mass of 40 mg. This was several orders of magnitude lower than expected to be released from the cell cytoplasm, likely due to poor cell lysis. Lysis of gram-negative cells, such as E. coli, is often performed with both 1 mg/mL of lysozyme and 2 mM EDTA, but the latter was not used to prevent protein denaturation (Birdsell and Cota-Robles 1967). This formulation is not likely to denature much if any of the CelA protein and so this method may be used in future work. Alternately, the cell pellet could be lysed by mechanical shear through a French press, or through repeated freezing and thawing cycles. Unfortunately none of these corrective measures were taken, and this severely limited the protein yield from this fermentation. However, this processing was valuable in establishing the purification scheme and producing a small but useful quantity of the fusion protein.

The induced soluble fraction, except for a saved sample of 100 μL, was passed through a 0.22-μm sterile filter and loaded onto the Ni–NTA column through the pump at 0.3 mL/min. The column was used with the previously described binding, wash, and elution buffers. The scales of the nonbinding and elution peaks were larger than the previous runs, due to the larger quantity of protein loaded onto the column, but the general binding characteristics remained the same (Figure 4). One important procedural note was that the soluble fraction was added directly to the column and, although this can be acceptable for small volumes, it is a poor practice and should avoided. A better practice to be used in future work is to dilute the sample with 2x binding buffer. The volume of the elution peak was 4.7 mL and both it and the nonbinding peak were saved.
Figure 4. CelA fusion protein purification on a Ni–NTA column. The broad peak A did not bind to the column, and peak B is the elution fraction. The column was equilibrated in binding buffer, loaded with sample, rinsed with binding, wash, and elution buffers, as shown on the UV280 chromatogram (right to left).

**Purification by gel filtration chromatography using P-100 resin**

A 1.5-cm diameter, 108-cm high gel filtration column of Bio–Gel P-100 resin (Bio–Rad) was equilibrated in 50 mM Tris–HCl, pH 8.0, for a day before use. The column was operated at room temperature at a flow rate at 6 mL/h, which was below the maximal recommended linear flow rate for this resin of 5 cm/h, corresponding to 8.9 mL/h for this column (Deutscher et al. 1990). The column head was stable with approximately 10 cm of buffer, but this was removed before loading the sample onto the column. The Ni–NTA elution fraction (4.2 mL) was loaded onto the column by gravity flow, and then the column head was restored and the pump was resumed. A single 12-mL protein peak began 15 h after loading the column, corresponding to approximately 90 mL of buffer exiting the column before protein elution, or approximately half of the total column volume. The total volume of buffer run through the column was 130 mL. The absorbance detector was turned off before the small proteins and salts, which including imidazole and NaCl, were recorded leaving the column.

**Concentration by ultrafiltration**

The collected elution peak from the P-100 run was concentrated and desalted using a 30 MWCO Vivaspin PES (Sartorius) spin column. The pooled elution peak was 12 mL, and all but 500 µL was pipetted into the spin column. It was centrifuged in a 25° fixed-angle rotor at 8,000g for 15 min following the manufacturer’s protocol, sufficient to reduce the volume to <500 µL. The concentrated solution was washed three times, each time by adding approximately 15 mL of 10 mM Tris–HCl buffer, pH 8.0, and then by concentrating through centrifugation as described above. This protocol removes approximately 98% of any contaminating salts, most of which will have already been removed by gel filtration. The final volume of the purified CelA fusion protein was 550 µL, with an estimated concentration of 3.2 µg/µL, determined by absorbance at 280 nm with the Nanodrop®. A more reliable test, BCA, gave the concentration as 0.8 µg/µL.

**2D gel electrophoresis analysis of CelA fusion protein purity**

Two-dimensional electrophoresis was conducted by the ISU Protein Facility with isoelectric focusing (IEF) performed on a 3–10 pI strip followed by gel electrophoresis on a 7-cm 12% acrylamide gel. The sample volume was 15 µL of the concentrated CelA fusion protein, for an expected mass of 50 µg, based on the UV280 absorbance. The IEF stage used an average voltage
of 4130 V and 29400 V-h total. The acrylamide gel was run with 180 V followed by staining with Coomassie Blue. Protein migration did not occur properly, possibly due to protein agglomeration, resulting in two bands forming, neither of which reached the expected size of 54 kDa. The ISU Protein Facility suggested that there was no indication that these two bands represented separate proteins but instead that the sample protein appeared pure within the detection limit of Coomassie Blue staining.

The PI of the bulk of the CelA fusion protein was estimated from the IEF strip as 6.15, and all of the protein was within the range of PI 6.05 and 6.19. The theoretical PI for the CelA fusion protein, based on the amino acid sequence, was estimated using an online bioinformatics tool (available at www.bioinformatics.vg/sms/protein_iep.htmL) as 5.44. For reference, the theoretical PIs for the entire CAC0915 protein and GH44 PfamB regions were 5.06 and 4.90, respectively.

**SDS–PAGE analysis of the CelA fusion protein purification scheme**

As previously mentioned, 500-μL samples of the fermentation fraction and purification samples were saved to evaluate the purification scheme by SDS–PAGE analysis, to quantify the protein concentrations by BCA, and to evaluate enzymatic activity by the tetrazolium blue assay (Jue and Lipke 1985, Smith et al. 1985). A SDS–PAGE gel was prepared with the preinduction and induction harvest (induced +4 h) samples, which were very similar to those shown in Figure 15. Samples from each purification step, and other fermentation fractions of interest, were diluted with 2x reducing buffer, each to 1x concentration, and all samples and standards were boiled for 4 min. The gel used was a precast 4–20% polyacrylamide gel (Bio–Rad), and this gel was run with the previous SDS–PAGE gel. Therefore the run and stain conditions are identical to those of previous runs.

Some important observations from this SDS–PAGE run can be made about the effectiveness of each step of the purification scheme (Figure 5). Although the contrast against the background is poor for soluble fraction lane, careful observation shows a clear presence of the target protein and more than a dozen other distinct proteins of similar intensity. The next purification stage was affinity chromatography with the Ni–NTA column, and this stage was effective in removing most of the contaminating proteins. The primary proteins that remained were approximately 90, 60, 25, and 10 kDa, as estimated using the broad range standard (Bio–Rad). The eluted peak from gel filtration, which unfortunately also shows poor contrast to the background, appears to have removed the 60- and 10-kDa proteins. Finally, ultrafiltration shows the final processed form of the protein, which still has 90- and 25-kDa contaminating proteins. This sample has been visualized two other times, by 2D gel electrophoresis and on the other SDS–PAGE gel. The 2D gel showed no other contaminating proteins with Coomassie Blue staining. Even the other SDS–PAGE gel appears to show essentially pure protein, and it was stained with both silver stain and Coomassie Blue. The difference between these two SDS–PAGE samples was that lane 16 was loaded with twice as much protein as lane 2 to detect these trace contaminating proteins. The CelA fusion protein appears to be >95% pure, based on these three instances of visualizing the protein, and since it required such gross overloading before the contaminants were visible. A final polishing step on an anion-exchange resin is recommended for future work before attempting to crystallize the CelA fusion protein.
Figure 5. CelA fusion protein (size marked) purification stages. 11) preinduction, 12) induced +4 h, 13) soluble fraction, 14) Ni–NTA elution peak, 15) P-100 elution peak, 16) purified protein, 17) osmotic shock, 18) concentrated broth, 19) broth permeate, 20) broad-range standard.

References

Attachment of Annexin V and Horseradish Peroxidase to Single-Walled Carbon Nanotubes

Luís F. F. Neves\textsuperscript{1,2}, Naveen R. Palwai\textsuperscript{1}, David E. Martyn\textsuperscript{1}, Yongqiang Tan\textsuperscript{1}, Daniel E. Resasco\textsuperscript{1}, and Roger G. Harrison\textsuperscript{1}

\textsuperscript{1}School of Chemical, Biological and Materials Engineering – University of Oklahoma
\textsuperscript{2}Faculty of Science and Technology and Faculty of Medicine – University of Coimbra (Portugal)

Abstract

To develop a therapeutic system with cancer cell selectivity, the present study proposes a possible specific and localized tumoral treatment. Phosphatidylserine exposure on the external face of the cell membrane is almost completely exclusive to cancer cells and endothelial cells in the tumor vasculature. With this knowledge and because of the fact that the protein annexin V has excellent Ca\textsuperscript{2+}-phospholipid binding properties, we have excellent conditions for the development of a therapeutic system.

In this study, we have evaluated the attachment of two proteins, horseradish peroxidase (HRP) and annexin V, to single-walled carbon nanotubes (SWNTs) by two different methods—adsorption and covalent attachment. HRP was adsorbed on SWNTs with a good protein loading (1.7 mg/mg) and almost full retention of enzyme activity. HRP was covalently attached to SWNTs with even higher protein loading (3.0 mg/mg) and with a relatively small loss (19\%) of native enzyme activity. Adsorption of annexin V on SWNTs results in complete precipitation of the SWNTs. However, the covalent attachment of annexin V to SWNTs gave high protein loading (5.1 mg/mg).

Introduction

Cancer can be defined as a group of diseases characterized by uncontrolled cell division, invading the adjacent or distant tissues (metastasis) (Sanchez 2004). Being considered the world’s most complex disease process, it is related to 100 or more single diseases (Fink 1979). There are several possible environmental factors that can induce cancer, such as exposure to radiation, hereditary factors, viruses, smoke, alcohol, dietetic habits, contributing to approximately 80\% of cancers (Fink 1979; Cooper 2004). Cancer can exhibit different symptoms, although it can be asymptomatic until advanced stages. Risk factors include geography, ethnics, dietary habits, age, and hereditary genes that increase the predisposition for cancer.

In order to treat cancer, there is an inherent difficulty in distinguishing malignant cells from normal cells in the organism. Both of them share the same origin, which explains the fact that cancer cells are not significantly recognized by the immune system.

Generally, cancer treatment results from the combination of different therapeutic techniques, such as surgery and chemotherapy (Sanchez 2004). According to some studies, these techniques can be very efficient; however, they carry associated-secondary effects (Sanchez 2004) for the patient. A specific case is chemotherapy, a therapy based on the introduction of chemical substances in the bloodstream in order to control cell processes. The secondary effects associated with this therapy are related to the collateral destruction of normal cells (Sanchez
When manifested, examples of these effects are anxiety, anemia and fatigue due to the red blood cells decreasing, diminution of white cells and platelets, renal perturbations, digestive system alterations and loss of appetite, increase in weight, alopecia, in addition to other symptoms.

In this study, a protein is used that has phosphatidylserine (PS)-binding properties – annexin V – and is a very important marker for cancer cells, since cancer cells and endothelial cells in the tumor vasculature have PS exposure on the external leaflet of the plasmatic membrane. The annexins are a group of proteins with similar properties (Smith, Kaetzel et al. 1990; Heizmann 1991) that were initially named by Geisow (Geisow 1986; Smith, Kaetzel et al. 1990). That name is derived from the Greek annex meaning “bring/hold together” (Benz and Hofmann 1997). The annexins are different proteins, with a common C-terminus attached to an amino acid repeated domain, although presenting a unique N-terminal domain (Schlaepfer and Haigler 1990; Smith, Kaetzel et al. 1990; Heizmann 1991; Benz and Hofmann 1997).

The attachment of annexin V to single-walled carbon nanotubes (SWNTs) will give a complex that can be used to target cancer cells and endothelial cells of the tumor vasculature, which have PS exposed on their cell surface. This association makes possible cancer cell destruction, using localized irradiation provided by near-infrared (NIR) radiation. The energy absorption by the nanotubes induces a temperature increase in the cancer cells culminating in the denaturation and loss of cellular functionalization. The specificity of treatment should result in minimal secondary effects to the normal cells.

Materials and Methods

Materials

Purified and freeze dried CoMoCAT SWNTs were provided by Southwest Nanotechnologies, Inc. Horseradish peroxidase (40 kDa) was obtained from Worthington Biochemical. Sodium cholate (NaC, from Sigma-Aldrich) and low-viscosity sodium carboxymethylcellulose (CMC, from Sigma-Aldrich) were used as dispersants. A horn sonic dismembrator (Cole Parmer, CPX 750) equipped with a microtip of 1/8" in diameter was used. The resulting suspension was centrifuged in an automatic centrifuge (IEC Multi, Thermo Electron Co.) at 25,000 g for 30 minutes. A characteristic dark-colored aqueous suspension was obtained as result of this sonication process. To perform the attachment of annexin V to SWNTs, 2-(N-morpholino)ethanesulfonic acid (MES), NaCl, 1-ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride (EDC), N-hydroxy-succinimide, 98% (NHS), 2-mercaptoethanol, hydroxylamine, sodium phosphate purchased from Sigma and a 100 kDa dialysis membrane (Spectra-Por) were used.

Expression and purification of annexin V

Recombinant annexin V (36 kDa) was expressed in E. coli from a plasmid that we previously constructed (Palwai 2007). Recombinant annexin V was purified from the cell lysate supernatant by metal affinity chromatography and freeze dried. The overall production of purified annexin V protein was 59 mg/L.
Adsorption of horseradish peroxidase on SWNTs

Sodium phosphate (20 mM) was mixed with the SWNT suspension (7 ml) (3 mg of 8L SWNT in 2% sodium cholate). Twenty mg of HRP were added to that solution. The solution was dialyzed against a 2 L sodium phosphate buffer (20 mM, pH 7.4, 12 h) using a 10 kDa dialysis membrane. After that time period, the solution was transferred to a 100 kDa dialysis membrane, which was dialyzed against a 2 L sodium phosphate buffer (20 mM, pH 7.4). The dialysis was performed during 8 h, with a buffer replacement after the first 4 h. The solution was centrifuged at 29,600 x g for 1 h. The protein in the solution was quantified performing the Bradford protein assay (Bio-Rad Laboratories).

A UV-Vis spectrophotometer (UV-2100 PC Shimadzu) was used to investigate the optical transitions in the wavelength range 300-900 nm, while a Bruker Equinox 55 FTIR was used for the NIR range, 850-1,350 nm. A UV-Vis-NIR spectrum was obtained, and it is presented in the Results section.

The HRP activity was quantified by performing an enzyme activity assay. The H$_2$O$_2$ is the initiator of the reaction reacting with the 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) that is the substrate. When enzymes are present in solution, those two reagents are converted to water and oxidized ABTS.

$$\text{H}_2\text{O}_2 + \text{ABTS} \underset{\text{Peroxidase}}{\longrightarrow} 2\text{H}_2\text{O} + \text{oxidized ABTS}$$

When in the presence of an enzyme with some activity, the reaction causes an increase of the absorbance, with a characteristic absorbance stabilization after the first minute, since the maximum linear rate occurs within the first minute of the reaction. This assay is based on the continuous spectrophotometric rate determination (25 °C, pH = 5.0, A$_{405\text{nm}}$, light path = 1 cm) method. The $\Delta$A$_{405\text{nm}}$/minute is calculated using the maximum linear rate for the test and blank samples. According to the definition, one unit will oxidize 1.0 µmole of 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) per minute at pH 5.0 at 25 °C.

The measurement was repeated after 5 days, and the solution during that period remained at room temperature. The activity and protein concentration were also measured in the dialysate, in order to investigate the possibility of some remaining protein in that solution.

The initial solution (SWNT dispersed into sodium cholate) and the final solution (SWNT-HRP complex) were analyzed by atomic force microscopy (AFM) using a Digital Instruments NanoScope III instrument in the tapping mode.

Adsorption of Annexin V on SWNTs

In order to evaluate the adsorption of annexin V on SWNTs, sodium phosphate (20 mM) was mixed with the SWNT suspension (1.75 ml) (3mg 8L SWNT in 2% sodium cholate). Five mg of previously prepared annexin V was added to that solution. The solution was dialyzed against a 500 ml sodium phosphate buffer (20 mM, pH 7.4, 12 h) using a 10 kDa dialysis membrane. After that time period, the solution was transferred to a 100 kDa dialysis membrane, which was dialyzed against a 2 L sodium phosphate buffer (20 mM, pH 7.4). The dialysis was performed during 8 h, with a buffer replacement after the first 4 h. The solution was centrifuged at 29,600 x g for 1h. The protein in the solution was quantified performing the Bradford protein assay (Bio-Rad).

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Attachment of HRP and annexin V to CMC-SWNTs using EDC and NHS

The attachment of HRP to SWNTs using EDC and NHS requires an initial 8 h dialysis (0.5 M NaCl), that removes the excess and unbounded CMC in the initial suspension (SWNT-CMC). The buffer was replaced after 4 h from the start of dialysis.

The procedure for two-step coupling of SWNT-CMC (3 mg 8L SWNT in 1% low viscosity CMC) and HRP using EDC and NHS is described here. The activation buffer (0.1 M MES (2-(N-morpholino)ethanesulfonic acid), 0.5 M NaCl, pH 6.0) was mixed with the 7 ml SWNT-CMC solution (estimated CMC concentration = 0.345 mg/ml).

The EDC and NHS were equilibrated to room temperature before opening the bottles. EDC (2.8 mg ~ 2 mM) and 4.2 mg of NHS were added to 7 ml of SWNT-CMC solution with the activation buffer reacting for 15 minutes at room temperature. In order to quench the EDC, 9.8 μl of 2-mercaptoethanol was added to a final concentration of 20 mM. The protein was added to the activated CMC at an equal weight ratio with CMC. The reaction took place at room temperature for 2 hours. To quench the reaction, hydroxylamine was added to a final concentration of 10 mM. This method hydrolyzes nonreacted NHS present on CMC and results in the regeneration of the original carboxyls. Other quenching methods involve adding 20-50 mM Tris, lysine, glycine or ethanolamine; however, these primary amine containing compounds modify carboxyls on CMC (Pierce Catalog, 2006).

In order to remove any excess reagent, a dialysis was performed using a 100 kDa dialysis membrane immersed in a 2 L sodium phosphate buffer (20 mM, pH 7.4). The buffer was replaced after 4 h from the start of dialysis, which lasted for a total of 8 h. The final volume inside the dialysis membrane was measured. A centrifugation was performed at 29,600 x g for 1 h, in order to isolate the SWNT-CMC fraction bound to the protein. The protein concentration in the final suspension (obtained after centrifugation) and in the dialysate was measured using the Bradford protein assay.

The HRP activity was quantified by performing an enzyme activity assay. The measurement was repeated after 5 days, with the solution held during that period at room temperature. The activity and protein concentration were also measured in the dialysate, in order to investigate the possibility of some remaining protein on that solution.

Results

Adsorption of HRP on SWNTs

To conserve annexin V, adsorption of the model protein horseradish peroxidase (HRP) directly on SWNTs was attempted first using an existing protocol. This protocol was optimized in order to maximize the yield of protein adsorption on SWNTs. Enzyme activity and protein concentration results for HRP adsorbed on SWNT are given in Table 1. The enzyme activity / enzyme activity of native protein (0.92) is similar to that obtained in a previous experiment (Palwai 2007). A good loading of protein on the SWNTs was achieved (1.7 mg/mg). Measurements taken after holding the SWNT-HRP suspension for 5 days at room temperature indicate a complete loss of enzymatic activity. The finding of no enzyme activity or protein in the final 2 L dialysis solution indicates that HRP was all adsorbed on the SWNTs.

The initial suspension (SWNT dispersed in 2 wt % sodium cholate) and the SWNT-protein suspensions before and after centrifuging were analyzed by UV-Vis-NIR spectroscopy (Figure 1). The nanotube concentrations (Table 2) were determined from the absorbance at 800 nm that corresponds to a valley in the absorbance spectrum where the absorbance is minimally
influenced by the specific $S_{11}$ transitions of the SWNT or by the adsorbed protein. From a direct analysis of the UV-Vis-NIR absorbance spectrum, it is possible to note that in the 300-400 nm wavelength range of the spectra, the absorbance was significantly greater for both of the samples with SWNT-protein than for SWNTs alone, and the SWNT-protein after centrifugation had significantly higher absorbance in this same range than the SWNT-protein before centrifugation. This latter difference is indicates that the SWNT-protein before centrifugation contained SWNTs with varying amounts of protein adsorbed per SWNT, and after centrifugation the SWNTs with lower amounts of protein adsorbed were removed.

A strong absorption band at 980 nm is present, which is a typical band for CoMoCAT samples and attributed to the $S_{11}$ transition of (6,5) nanotubes. The intensities of the peaks were similar for the pristine SWNTs and the SWNT-protein pre-centrifugation samples. However, there is a slight, but consistent red shift in the SWNT-protein samples that can be explained as a stronger SWNT-protein interaction than with the surfactant.

The atomic force microscopy images (Figure 2) present strong evidences that corroborate the success of this experiment. The arrows indicate the adsorption of sodium cholate (Figure 2 a) and HRP (Figure 2 b). The height of the aggregates of HRP in the final suspension (3.8-6.0 nm) is considerably higher than those of sodium cholate (1.0-1.5 nm), and this fact is consistent with the size of similar proteins that have about the same molecular weight as HRP. For example, $\beta$-lactoglobulin has a 35 kDa molecular weight and a diameter of 5.4 nm (Palwai, Martyn et al. 2007).

**Table 1:** Enzyme activity and protein concentration results for HRP adsorbed on SWNTs.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Enzyme activity, U/mg protein</th>
<th>Protein concentration, (mg/L)</th>
<th>Enzyme activity/ enzyme activity of native protein</th>
<th>SWNT Concentration, (mg/L)</th>
<th>Protein Weight, SWNTinish Weight (mg/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SWNT-protein suspension after centrifugation</td>
<td>Day 0</td>
<td>189</td>
<td>266</td>
<td>0.92</td>
<td>153</td>
</tr>
<tr>
<td></td>
<td>Day 5, held at room temperature and centrifuged again</td>
<td>0</td>
<td>16</td>
<td>0</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Final dialysis solution (2 L) using 100 kDa membrane</td>
<td>0</td>
<td>0</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

**Table 2:** SWNT concentrations (as measured by the absorbance at 800 nm) for the study of HRP adsorbed on SWNTs.

<table>
<thead>
<tr>
<th>Compound</th>
<th>SWNT concentration, mg/L</th>
<th>Yield, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pristine SWNTs</td>
<td>194</td>
<td>100.0</td>
</tr>
<tr>
<td>SWNT-protein, pre-centrifugation</td>
<td>174</td>
<td>89.6</td>
</tr>
<tr>
<td>SWNT-protein, post-centrifugation*</td>
<td>153</td>
<td>78.9</td>
</tr>
</tbody>
</table>
Figure 1: UV-Vis-NIR absorbance spectrum (normalized) for study of HRP adsorbed on SWNTs. A UV-Vis spectrophotometer (UV-2100 PC Shimadzu) was used to investigate the optical transitions in the wavelength range 300-900 nm while a Bruker Equinox 55 FTIR was used for the NIR range, 850-1,350 nm.

Figure 2: AFM images showing SWNT with sodium cholate and HRP protein for the study of HRP adsorbed on SWNTs. (a) SWNT in sodium cholate after sonication and centrifugation. Arrows indicate solid sodium cholate with a height of 1.0-1.5 nm (does not include 0.8 nm
SWNT height). (b) SWNT-protein after dialysis and centrifugation. Arrows indicate protein associated with SWNT. The height is 3.8-6.0 nm (does not include 0.8 nm SWNT height).

**Adsorption of Annexin V on SWNTs**

In this study, we tried to perform the adsorption of annexin V on SWNTs, using the same protocol used for the HRP protein. However, during the experiment and after the final dialysis, the SWNT completely precipitated (formation of nanotube aggregates). After the centrifugation, the soluble fraction was completely clear, showing the absence of nanotubes associated with the annexin V in suspension.

With this important result, new methodologies were searched in order to make it possible to attach the protein to SWNTs. Since Dr. Daniel Resasco’s group had already shown that SWNTs could be suspended using carboxymethylcellulose (CMC), the coupling of annexin V to the CMC-SWNT complex was evaluated. This result led to the development of a new protocol, using a one-step EDC reaction with carboxyl and amine-containing molecules.

**Attachment of HRP to CMC-SWNTs using EDC and NHS**

The attachment of the HRP protein to the CMC-SWNTs complex was evaluated using EDC and NHS, according to the protocol presented in the methods section. The SWNT-CMC-HRP complex formed a stable suspension after dialysis and centrifugation. The results for this reaction are presented on Tables 3 and 4. The enzyme activity for the SWNT-CMC-protein suspension after centrifugation was 167 U/mg. The ratio between the enzyme activity and the enzyme activity of the native protein was 0.81, which indicates that there was some loss of activity compared to the native enzyme. The ratio between protein weight and SWNT weight (protein loading) was 3.0. The conjugation of SWNT-CMC with HRP using EDC was successful, with an increase of 76% of protein loading compared to the adsorption experiment. No enzyme activity or protein was found in the final dialysis solution (2 L), which indicates that unbound HRP had been removed by the dialysis.

**Attachment of Annexin V to CMC-SWNTs using EDC and NHS**

Annexin V was attached to CMC-SWNTs using EDC and NHS by the same methods that were used to attach HRP (see results in Tables 5 and 6). The SWNT-CMC-annexin V complex formed a stable suspension after dialysis and centrifugation. Although the protein and SWNT concentrations were lower than when HRP was used, the protein weight/SWNT weight was 70% higher.
Table 3: Enzyme activity and protein concentration results for the study of HRP attached to CMC-SWNT using EDC and NHS.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Enzyme activity, U/mg protein</th>
<th>Protein concentration, mg/L</th>
<th>Enzyme activity/enzyme activity of native protein</th>
<th>SWNT Concentration, mg/L</th>
<th>Protein Weight, mg/mg SWNT Weight</th>
</tr>
</thead>
<tbody>
<tr>
<td>SWNT-CMC-HRP suspension after centrifugation</td>
<td>167</td>
<td>239</td>
<td>0.81</td>
<td>81</td>
<td>3.0</td>
</tr>
<tr>
<td>Final dialysis solution (2 L) using 100 kDa membrane</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>

Table 4: SWNT concentrations (as measured by the absorbance at 800 nm) for the study of HRP attached to CMC-SWNT using EDC and NHS.

<table>
<thead>
<tr>
<th>Compound</th>
<th>SWNT concentration, mg/L</th>
<th>Yield, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pristine SWNTs</td>
<td>320</td>
<td>100.0</td>
</tr>
<tr>
<td>SWNT-protein, pre-centrifugation</td>
<td>290</td>
<td>135.9</td>
</tr>
<tr>
<td>SWNT-protein, post-centrifugation*</td>
<td>81</td>
<td>52.7</td>
</tr>
</tbody>
</table>

Table 5: Protein concentration results for the study of annexin V attached to CMC-SWNT using EDC and NHS.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Protein concentration, mg/L</th>
<th>SWNT Concentration, mg/L</th>
<th>Protein Weight, mg/mg SWNT Weight</th>
</tr>
</thead>
<tbody>
<tr>
<td>SWNT-CMC-annexin V suspension after centrifugation</td>
<td>74.</td>
<td>14.6</td>
<td>5.1</td>
</tr>
<tr>
<td>Final dialysis solution (2 L) using 100 kDa membrane</td>
<td>0</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>
Table 6: SWNT concentrations (as measured by the absorbance at 800 nm) for the study of annexin V attached to CMC-SWNT using EDC and NHS.

<table>
<thead>
<tr>
<th>Compound</th>
<th>SWNT concentration, mg/L</th>
<th>Yield, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pristine SWNTs</td>
<td>26.6</td>
<td>100.0</td>
</tr>
<tr>
<td>SWNT-protein, pre-centrifugation</td>
<td>25.6</td>
<td>96.2</td>
</tr>
<tr>
<td>SWNT-protein, post-centrifugation*</td>
<td>14.6</td>
<td>54.9</td>
</tr>
</tbody>
</table>

Discussion

SWNTs can be exploited as molecular transporters for various cargos. The biocompatibility, unique physical, electrical, optical, and mechanical properties of SWNTs provide the basis for new classes of materials for drug, protein, and gene delivery applications.

Evaluating the results, the adsorption of HRP on SWNTs was successful using the two different attachment methods. When HRP adsorption was performed using EDC as a crosslinker agent, there was some loss of activity compared to the native enzyme (enzyme activity/enzyme activity of native protein = 0.81). However the protein weight/SWNT weight was 76% higher than when HRP was attached by adsorption.

Although it was not possible to adsorb annexin V to SWNTs and keep the SWNTs suspended, the attachment of annexin V to SWNT-CMC using EDC and NHS was successful. Using this covalent attachment method, there was an increase of 70% of protein loading compared to the same experiment using HRP. The binding of the SWNT-CMC-annexin V complex to PS will be evaluated in future studies.

The results obtained in the present study using annexin V and HRP provide encouragement that we can develop a therapeutic system using targeted SWNTs to treat cancer.

References


Metal/polymer composite membranes for low trans fatty acid hydrogenation of soybean oil

D. Singh, M. E. Rezac, P. H. Pfromm*

Department of Chemical Engineering, Kansas State University
1005 Durland Hall, Manhattan, Kansas, 66506-5102, USA

Abstract

Partial hydrogenation of vegetable oils is carried out to improve the chemical stability and raise the melting point to produce semi-solid products such as margarine. Trans fatty acids formed during traditional hydrogenation have come under intense scrutiny in regard to human health. Here we report partial hydrogenation of soybean oil using a high performance integral-asymmetric polyetherimide membrane sputtered with platinum to deliver hydrogen directly to or near the catalytic sites. Oil flows past the platinum-coated "skin" side of the membrane while dissolved molecular and some atomic hydrogen is supplied from the highly porous substructure of the membrane. The membrane has a high hydrogen flux but is essentially impermeable to soybean oil. Hydrogenation using our metal/polymer catalytic composite membrane produced 17 wt% less trans fatty acids than the conventional nickel on silica catalyst. Our concept requires hydrogen pressures of only about 50 psig and temperatures near 70°C. The polymeric base membranes used here have been mass produced and can be packaged in spiral wound modules. The relatively mild reaction conditions and the direct pathway to produce useful membrane modules combine to make our concept promising for near-term application.

* to whom correspondence shall be addressed, e-mail pfromm@ksu.edu, phone 785-532-4312, fax 785-532-7372

Introduction

Hydrogenation of vegetable oil to improve its oxidative stability and increase its solid fat content has been practiced since the early 19th century. Improved oxidative stability increases the shelf life and the solid fat content makes it more suitable for margarines, shortening, and frying applications. Recent health concerns regarding trans fatty acids (TFAs) formed during hydrogenation have led to a renewed interest in hydrogenation technologies that minimize trans fatty acids. The optimum technology should be able to hydrogenate linolenic acid (18 carbon atoms, three double bonds, abbreviated as C18:3, or Ln) as it is very easily oxidized, maintain lenoleic acid (C18:2, L) which forms an essential part of the human diet, and minimize the formation of TFA and stearic acid (saturated, C18:0, S) which are a cause of health concerns. The technology being considered should be easy to commercialize and be compatible with the existing hydrogenation facilities. Processing temperatures and hydrogen pressures compatible with existing capital equipment are especially important.

Industrial hydrogenation of vegetable oil is a three-phase (gas-liquid-solid) process carried out in a batch autoclave over Ni based catalyst as a slurry at 110-190 °C, 30-70 psi, and 0.01-0.15 wt%
nickel. Molecular hydrogen has to dissolve in the oil, diffuse through the liquid boundary layer surrounding the catalyst particles towards the catalyst surface and adsorb on the catalyst surface where the hydrogen dissociates into adsorbed hydrogen atoms. These hydrogen atoms react with double bonds in the fatty acid molecules on the surface of the catalyst. The reactions and reaction steps involved in the catalytic hydrogenation of vegetable oil are discussed by Dijkstra et al. using the Horiuti-Polanyi mechanism. If the availability of adsorbed hydrogen atoms at the surface of the catalyst is high, the hydrogenation tends towards the formation of desirable cis fatty acids. On the other hand, if the availability of adsorbed hydrogen atoms at the surface of the catalyst is low, hydrogenation shifts towards the formation of undesirable trans fatty acid (TFAs). If hydrogenation is allowed to proceed too far then undesirable completely saturated oil is obtained. The balance between available atomic hydrogen at the catalyst surface, availability of oil molecules with double bonds, and the associated mass transfer processes determines the outcome of the process. Here we attempt to solve the issue of hydrogen starvation of the catalyst surface by supplying hydrogen directly at or near the catalytic sites through active (pressure-driven) diffusion of hydrogen through an asymmetric metal/polymer composite membrane.

In the conventional hydrogenation slurry reactors, due to significant gas-liquid mass transfer limitations, the surface of the catalyst often may experience hydrogen starvation, which results in increased amounts of trans fatty acids. The relatively high temperature used in conventional reactors to promote hydrogenation also promotes the formation of TFAs by increasing the hydrogenation consumption rate which exacerbates hydrogen starvation of the catalyst surface. TFAs can be minimized by increasing pressure, decreasing temperature, and increasing agitation to address mass transfer limitations. All these changes in process conditions increase the hydrogen availability at the surface of the catalyst and lead to lower TFA. However, there are practical limits to which pressure and agitation can be increased and thereby TFA reduced in conventional reactors.

Precious metal catalysts are active at considerably lower temperatures and can thus potentially produce less TFA as compared to conventional Ni catalysts. The order of catalytic activity of precious metal catalysts is Pd > Rh > Pt > Ru. The cis-trans isomerisation follows the order Pd > Rh > Ru > Pt with Pt having the least cis-trans isomerisation selectivity. Another disadvantage of using metal catalysts in the form of solid particles with metal deposits on their surface in slurry systems is the difficult separation of the catalyst particles from the reaction mixture which results in loss of catalyst. This has prevented the use of more expensive precious metal catalysts in slurry systems.

Hydrogenation of soybean oil with our metal/polymer asymmetric composite membranes offers the advantages of reduced mass transfer limitations, operation at low temperature through the use of immobilized precious metals, and no loss or need for recovery of catalyst particles. Several authors have studied the hydrogenation of sunflower oil using membranes in the pore-flow-through mode with the aim to reduce the TFA formation. In these systems oil saturated with hydrogen is pumped through the pores of catalytically active porous membranes (catalyst immobilized in the membrane pores) with the convective flow in the pores minimizing mass transfer limitations. However the availability of hydrogen at the surface of the catalyst may be further increased and thus TFA formation reduced further if hydrogen is supplied directly at the surface of the catalyst as in the work proposed here. Gryaznov et. al. studied the concept of
supplying hydrogen at the catalyst surface for the hydrogenation of cyclopentadiene. A polydimethylsiloxane film was covered with a dense layer of Pd-Ru alloy which supplied atomic hydrogen at the surface of the catalyst for the hydrogenation reaction. The very low hydrogen permeability of metals, however, does limit the efficiency of this process. Here we demonstrate the hydrogenation of soybean oil over a “defective” metal/polymer composite asymmetric membrane with Pt catalyst deposited on the “skin” side of an integral-asymmetric polymeric membrane, Hydrogen is supplied directly at or near the surface of the catalyst. The Pt catalyst layer in our case is not perfect and has defects which is beneficial for the process as is discussed below. The hydrogenation results obtained are compared with existing and other developing technologies.

The polymer/metal composite catalytic membrane used here consists of an integral asymmetric polyetherimide (PEI) membrane with high flux and selectivity for hydrogen and negligible permeability to vegetable oil. Integral asymmetric PEI membrane were manufactured in our laboratory using a published method. The membrane consists of a highly porous substructure with a thin (approximately 0.2 microns) dense and defect-free layer known as the membrane skin which results in high hydrogen selectivity and flux for defect-free membranes. Gases permeate through the thin skin by the well-known solution/diffusion mechanism while the porous substructure allows for convection. The membrane skin is coated with a very thin layer of platinum using a magnetron sputter. Figure 1 shows a proposed schematic of our vegetable oil hydrogenation process. Hydrogen is supplied from the porous side of the membrane and oil is pumped across the skin/platinum side of the membrane where it comes in contact with the catalytic metal. A positive hydrostatic pressure is maintained on the oil side to prevent the membrane from being mechanically destroyed.

![Diagram of vegetable oil hydrogenation](image)

Figure 1: Schematic of vegetable oil hydrogenation as it would take place in the membrane reactor.

Hydrogen would emerge only as atomic hydrogen at the oil side of the metal coating if not only the polymeric skin but also the platinum layer were defect free. This would result in quite low hydrogen gas fluxes which is not what we observe. Due to the significant hydrogen fluxes measured with our sputtered membranes (see below) we assume that numerous defects in the metal layer are present. This, however, is neither unexpected nor detrimental to our concept.

We assume that the hydrogen diffuses mainly through the defect-free polymeric skin under the driving force of a hydrogen partial pressure difference maintained by consumption of hydrogen by hydrogenation near the membrane surface. Since the hydrogen is supplied near or at the catalytic sites and in part as atomic hydrogen permeating metal-coated portions of the membrane we expect that TFAs will be reduced since hydrogen starvation of the catalytic surface can be avoided. If hydrogen builds up in the oil then the driving force for hydrogen permeation will...
diminish which represents a type of self-limiting mechanism for hydrogen transport through the membrane to the oil phase.

In summary, we propose to minimize TFA formation by avoiding hydrogen starvation of the catalyst surface through directly supplying hydrogen to the catalytic sites via "reverse permeation" (from substructure to skin) of a high-flux thin film asymmetric metal/polymer composite membrane with a perfect polymer skin and a "defective" metal coating.

**Experimental procedures**

Soybean oil (Iodine Value IV = 127) was obtained from MP Biomedical (Solon, OH). The fatty acid profile as measured by us is 16:0, 11.5 wt%; 18:0,6.5 wt%; 18:1,29.1 wt%; 18:2, 44.8 wt%; 18:3,5.3 wt%; TFA, 1.4 wt% with the balance being 14:0 – 24:0. PEI to cast asymmetric membranes was obtained from General Electric (Huntersville, NC, Ultem-1000). Acetic acid (HPLC grade), acetone (99.5%), p-xylene (99.9%), and dichloromethane (99.9%), were obtained from Fisher Scientific (Rochester, NY), 1,1,2,2-tetrachloroethane (98%), and, a 5 wt% Platinum on activated carbon catalyst used in some hydrogenation experiments were from Sigma Aldrich (St. Louis, MO). A platinum target (99.95wt% platinum) for membrane sputtering was from Ted Pella Inc. (Redding, CA).

The integral asymmetric PEI membranes used in this study were fabricated using the phase inversion process as described by Peinemann. Circular stamps (4.6 cm diameter) are cut and tested in a filter holder (see below) using a bubble-flowmeter for their gas flux. The normalized gas flux and the ideal gas selectivity are calculated as a quality control. The ideal gas selectivity $\alpha_{A/B}$ is the ratio of the normalized gas fluxes for gas A and B at a given temperature and feed pressure.

The hydrogen flux of these membranes can be as high as 100 GPU (one gas permeation unit or GPU equals $10^{-6}$ cm$^3$ (STP) cm$^{-2}$ s$^{-1}$ cmHg$^{-1}$) and ideal gas selectivities measured by single gas permeation of $\alpha_{H_2/N_2}$ in the range of 40-200. Membranes were deemed acceptable with a hydrogen flux of at least 20 GPU and $\alpha_{H_2/N_2}$ of at least 40 at 50 psig feed pressure and room temperature before sputtering with platinum. The yield of acceptable membranes from a given hand-cast sheet was very good, on the order of 5 membranes per 6 by 5 inch sheet. Acceptable asymmetric membranes were stored in air. Before use in hydrogenation experiments the membranes were sputtered on the skin side with platinum using a DESK II magnetron sputter (Denton Vacuum, Moorestown, NJ, 3 seconds at 45 mA, 100 mtorr) and were re-tested for their gas transport properties. The gas flux of the membranes is reduced by on the order of 4-5 fold after sputtering as can be expected from coverage of the membrane with a metal layer containing defects. Gas selectivities after sputtering also change and the change depends on factors such as initial $\alpha_{H_2/N_2}$, and the defects on the platinum sputtered layer. Gas selectivities $\alpha_{H_2/N_2}$ of at least 20 after sputtering were deemed acceptable.
The platinum sputtered membrane was installed in a stainless steel 47 mm filter holder (model XX4404700, Millipore Corp., Billerica, MA). Before the start of the hydrogenation reaction, the Pt catalyst on the membrane was reduced using hydrogen at 50 °C for at least 15 hours applied to the platinum (skin) side of the membrane with the permeate side open to the atmosphere. A Parr Reactor (160 mL) was used as the reservoir for oil at 70 °C and this temperature was maintained in the entire system including the membrane reactor. The reactor was always stirred (standard four blade turbine type impeller, 100 rpm). To avoid oil oxidation, air was removed from the reactor with a nitrogen purge before heating the oil. 50 g of soybean oil was used in all experiments and was circulated with a flow rate of about 25 ml/min across the platinum sputtered side of the membrane using a gear pump (series GA, Micropump Inc., Vancouver, WA). The oil side pressure was maintained at 60 psig using ultra high purity (UHP) nitrogen applied to the headspace of the Parr reactor. UHP hydrogen was supplied from the porous substructure side of the membrane at 50 psig.

Two tests are reported here using the metal/polymer composite membranes. In one of these runs, a single membrane was used having a hydrogen flux of 1.3 GPU ($\alpha_{H_2/N_2} = 20$) measured after platinum sputtering. In the other test, three membranes were used in series having hydrogen fluxes of 5.5 ($\alpha_{H_2/N_2}=142$), 5.5 ($\alpha_{H_2/N_2}=47$) and 3 ($\alpha_{H_2/N_2}=60$) GPU measured after platinum sputtering. These three membranes would provide a hydrogen flow of $1.7 \times 10^{-4}$ moles/min as compared to $1.6 \times 10^{-5}$ moles/min provided by the single membrane of flux 1.3 GPU.

To compare our membrane hydrogenation concept with the conventional slurry catalyst system under similar conditions, a "modified conventional" run with 0.02 gram of 5 wt% Pt on activated carbon was performed. The catalyst was added directly to the oil in the Parr reactor. The oil was circulated through the membrane holder and hydrogen was supplied from the substructure side of a membrane (not platinum-sputtered) with a membrane having hydrogen flux of 3 GPU.
Another run was performed in "conventional" mode using 0.05 gram of 5 wt% Pt on activated carbon catalyst as a slurry batch reactor stirred at 200 rpm with 50 psig hydrogen applied to the headspace of the reactor.

Oil samples were converted to their corresponding fatty acid methyl esters (FAMEs) following the alternate method in AOCS official method Ce 2-66. FAMEs thus obtained were analyzed by gas chromatography (GC) using a 100 m CP-Sil 88 column in a Hewlett-Packard 6890 series gas chromatograph. AOCS official method Ce 1h-05 was followed for the analysis of fatty acids. Injection port and column were maintained at 250 °C and 181 °C, respectively, helium carrier gas at 1 ml/min and split injection (split ratio 1:100) was used. The IV of hydrogenated oil was calculated from the composition obtained by GC analysis using equation (1):\(^1\)

\[
IV = (\% C_{16:1} \times 0.9502) + (\% C_{18:1} \times 0.8598) + (\% C_{18:2} \times 1.7315) + (\% C_{18:3} \times 2.6152) \tag{1}
\]

If the hydrogenation of soybean oil is represented by the first-order irreversible reaction scheme as shown in equation (2) then it is possible to calculate hydrogenation selectivities (linolenate selectivity \(S_{Ln} = k_1/k_2\), linoleate selectivity \(S_L = k_2/k_3\), and, oleate selectivity \(S_O = k_1/k_3\)) using the experimental composition data from our experiments\(^12\).

\[
k_1 \quad k_2 \quad k_3
C_{18:3}(Ln) \rightarrow C_{18:2}(L) \rightarrow C_{18:1}(O) \rightarrow C_{18:0}(S) \tag{2}
\]

where \(k_1, k_2, k_3\) are pseudo-first-order rate constants.

Results and Discussion

An improved soybean oil hydrogenation process must not only significantly reduce the formation of TFA at comparable levels of hydrogenation as represented by the target IV number but the process must ideally do so at hydrogen pressures and operating temperatures that do not require major modifications to existing facilities. The need for costly catalyst materials must be minimized and long catalyst life is desirable. Below we show a comparison of our metal/polymer catalytic integral-asymmetric membrane process with conventional and other novel approaches.
Figure 3: Using a traditional platinum-on-carbon catalyst as suspended particles while supplying hydrogen through a polymer membrane results in the highest TFA values at a given IV. The catalytic membrane test with the highest H$_2$ flux per unit area of membrane results in lowest amount of TFA. (70 °C, membrane: 60 psig oil side pressure, 50 psig H$_2$ as hydrogen side pressure)

Figure 3 compares the TFA vs. IV profile obtained for three different hydrogenation experiments. It should be kept in mind that hydrogen starvation of the catalyst surface results in increased TFA formation. The modified conventional run (dissolved hydrogen supply into the oil via a not metal sputtered polymer membrane, nominal 3 GPU H$_2$, conventional platinum catalyst on carbon as particles suspended in the bulk oil) produced the most TFA while the catalytic membrane hydrogenation (platinum sputtered polymer membrane) with three individual membranes (having hydrogen fluxes of 5.5, 5.5 and 3 GPU, respectively measured after sputtering) produced the least TFA at a given IV. Even the "worst case" membrane hydrogenation with a relatively low hydrogen supply (nominal only 1.3 GPU H$_2$) produced less TFA than the modified conventional run. We hypothesize that our concept of supplying hydrogen from the substructure of the metal/polymer composite membrane directly at or near the catalytic sites reduces TFA formation due to alleviating hydrogen starvation of the catalyst surface when compared to supplying hydrogen by diffusion from the bulk oil phase. This is also supported by the fact that the membrane-catalyzed test with the lower amount of hydrogen supplied per unit area of the membrane shows a somewhat increased TFA production.
Figure 4: Composition of C18:0, C18:1, C18:2, C18:3 fatty acids with Iodine value for Pt slurry reactor (70 °C), Pt membrane reactor (70 °C, 14 GPU), and Ni slurry reactor (150 °C).

Figure 4 shows the composition profiles (C18:0, C18:1, C18:2, C18:3) for hydrogenation using catalytic membranes (hydrogen fluxes of 5.5, 5.5 and 3 GPU), a Pt on carbon catalyst (Pt/C) as a slurry at 70°C, and a nickel on silica (Ni/Si) catalyst as a slurry at 140°C. The composition profiles of C18:2 and C18:3 for the catalytic membrane are quite similar to that of the Ni/Si slurry reactor. The Ni/Silica catalyst appears to produce more C18:1 and less C18:0. The differences in composition profiles with increasing hydrogenation for the conventional slurry catalysts are characteristics of Ni and Pt catalysts and are consistent with the literature. The Pt/C slurry reactor and Pt in form of the sputtered catalytic membrane have very similar concentration profiles but the catalytic membrane produces about 19% less saturates as compared to the Pt/carbon slurry under similar conditions and at the same IV. The small differences in the composition profiles/hydrogenation selectivity may be attributed to the physical characteristics of the catalyst as it controls the diffusion of reactants in and out of the catalyst pores. Selectivity also depends on the availability of hydrogen at the surface of the catalyst. The selectivity increases with decreasing hydrogen availability at the surface.

Table 1 compares the selectivities obtained with the catalytic membrane to that of different catalysts in a slurry reactor and to novel processes being studied by others. The linolenic selectivity, SLN, is almost identical for the slurry reactors and our catalytic membrane but slightly higher than two other novel approaches. The TFA formed with the catalytic membrane reactor is only 3 wt% while the Ni/Pd slurry reactors produce about 20wt%. The very low linoleic selectivity of the membrane reactor as compared to slurry reactors indicates that the amount of oleic acid produced is less than for slurry systems (29wt% in membrane reactor as opposed to 51wt% in Pd/C slurry reactor at similar IV). The oleic selectivity, SO, for all the new systems being studied is lower than the conventional slurry based systems which means that more undesirable stearic acid (C18:0) is produced in these systems which is expected because factors that lead to lower trans selectivities also generally lead to lower hydrogenation selectivities.
Table 1: Selectivities and TFA reported using different catalysts and hydrogenation reactors.

<table>
<thead>
<tr>
<th>Process</th>
<th>IV</th>
<th>$S_{1n}$</th>
<th>$S_l$</th>
<th>$S_o$</th>
<th>Trans, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ni, slurry reactor, 140 °C, 44 psig$^{13}$</td>
<td>110</td>
<td>2.0</td>
<td>11.1</td>
<td>22.3</td>
<td>20</td>
</tr>
<tr>
<td>Pd/C, slurry reactor, 120 °C, $^{16}$</td>
<td>114</td>
<td>2.3</td>
<td>8.8</td>
<td>19.8</td>
<td>19</td>
</tr>
<tr>
<td>Pt-SiO$_2$, slurry reactor, 150 °C, 1 atm $^{17}$</td>
<td>117</td>
<td>2.7</td>
<td>2.9</td>
<td>7.6</td>
<td>7.8</td>
</tr>
<tr>
<td>PEM reactor, Pd-black cathode, 70 °C, 130 psig$^5$</td>
<td>115</td>
<td>1.5</td>
<td>1.6</td>
<td>2.4</td>
<td>11.8</td>
</tr>
<tr>
<td>Pd monolith catalyst, 80 °C, $^{18}$</td>
<td>103</td>
<td>1.4</td>
<td>1.0</td>
<td>1.3</td>
<td>8.5</td>
</tr>
<tr>
<td>Membrane Reactor (14 GPU flux), Pt, 70 °C</td>
<td>117</td>
<td>1.87± 0.03</td>
<td>1.37± 0.01</td>
<td>2.55± 0.02</td>
<td>3</td>
</tr>
</tbody>
</table>

Conclusions

This study demonstrates a novel integral-asymmetric metal-polymer composite catalytic membrane approach for low trans fatty acid hydrogenation of soybean oil. Hydrogen is supplied directly at or near the surface of an integral-asymmetric polymeric membrane sputtered with platinum by pressurizing the porous substructure of the membrane with hydrogen. The oil flows over the platinum-sputtered feed (skin) side of the membrane. No catalyst recovery from the oil is needed since the catalyst is immobilized on the membrane. The system is compatible with existing commercial hydrogenation facilities as far as temperatures (~70°C) and hydrogen pressure (~50 PSIG). Our approach shows significantly lower TFA values at comparable hydrogenation levels. We hypothesize that this is due to avoiding hydrogen starvation of the catalyst surface by rapid hydrogen permeation through a high performance asymmetric membrane.

![Figure 5](image_url)  
Figure 5: Effect of different catalysts/systems on trans fatty acid and saturates production during hydrogenation of soybean oil.
Acknowledgements

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References

Evaluation of the Convective Flow Decellularization for the Preparation of Biological Scaffold

Carolina Villegas Montoya* and Peter S. McFetridge
School of Chemical, Biological and Materials Engineering
University of Oklahoma
Norman, Oklahoma

ABSTRACT

Introduction: biological derived structures are being researched for use as scaffolds in tissue engineered graft development. Prior to implantation cellular components and immune reactant molecules need to be removed. Although current chemical and enzymatic treatments appear to be efficient in the majority of cases, improvements are needed to treat complex structures. The aim of this study was to introduce the convective flow decellularization method as an alternative to produce acellular scaffolds for tissue engineering applications. Materials and methods: Veins were dissected from human umbilical cords (HUV) and decellularized using agitation and convective flow methods. Convective flow decellularization was carried out at 50-mmHg trasmural pressure. All experiments were followed up to 72 hours and phospholipids and total protein extraction were assessed. Histological and SEM analyses of acellular HUV were performed. Results: Phospholipids and total proteins removed with convective flow were notably higher than agitation. After 3 hours of treatment, convective flow had removed more phospholipids and proteins than the total amount extracted with agitation method. Convective flow resulted in total decellularization while preserving the integrity of the extracellular matrix. Conclusions: Convective flow was successful at decellularizing a complex structure like a blood vessel, and the histological images showed that the collagen matrix had been retained. Further investigations are being developed to combine decellularization and reseeding processes with allogous cells within the same bioreactor, then, reducing graft handling prior to implantation.

*Corresponding Author: School of Chemical Biological and Materials Engineering, University of Oklahoma, 100 E Boyd, Norman, OK 73019-1004. Phone (405)420-8357. Fax (405)325-5813. Email: carolina@ou.edu

INTRODUCTION

Ex-vivo tissue is a promising alternative to artificial scaffolds; however, before implantation additional preparation demands an efficient extraction of cellular and nuclear components. Detection of these molecules by the host can induce an inflammatory or chronic immune response leading to the rejection of the implant [1]. Ex-vivo materials, including acellular dermis, amniotic membrane, small intestinal submucosa (SIS), and heart valves, have been researched as alternatives to synthetic matrices with an overall positive response for host cell migration and angiogenesis [2].

There is extensive research on chemical and enzymatic methods, but only slightly investigated is the development of different physical mechanisms to obtain an acellular matrix. The traditional decellularization processes are completed by submerging tissue in the different solutions under
simple incubation or mechanical agitation [3]. Efficiency of cell removal with chemical treatments under simple agitation becomes increasingly limited for complex tissue structures [4]. Furthermore, physical agitation gives highly variable total decellularization and therefore unsatisfactory results [5-7]. Physical treatments, like pressure-based methods, have been commonly used for tissue layer removal and as a mechanical force for cell lyses. The urinary bladder matrix is prepared by using intralumenal water under pressure to separate the muscle layer from the tunica submucosa [1].

We used human umbilical veins dissected from umbilical cords to evaluate the effects of pressure-based decellularization. The circulation of a decellularization solution through the inner side of the vein generates a convective or radial flow dragged by the transmural pressure differential. This convective flow increases the flux of solution through the tissue membrane, enhancing cell lyses, cellular antigens and phospholipids solubilization and removal while preserving ECM structure. Bioreactors can be used alternatively for decellularization procedures. These fulfill the requirements for specific tissue morphology and with slightly modifications can be used for decellularization purposes.

Our research aim was to assess a novel mechanism where physical forces and a delivery system are combined to produce an acellular biological matrix for use in tissue-engineered constructs. Although we used a cylindrical-like tissue, the principles of the mechanism apply to any other tissue shape as long as the used device fulfills the sample morphology requirements while achieving more efficient results than the mechanical agitation.

MATERIALS AND METHODS

*Human umbilical vein (HUV) isolation:* Human umbilical veins were isolated using an automated dissection as previously described [8]. Briefly, human umbilical cords were collected from the local hospital up to 48 hours after delivery. Upon arrival they were rinsed with water and cut in 120 mm segments. Cords were mounted on a stainless steel mandrel (6 mm OD by 200 mm length) and secured at both ends with nylon cable ties. Cords were gradually frozen to -80 °C for at least 12 hours to ensure a uniform temperature throughout the vessel wall. HUVs were dissected using an automated lathe (Central Machinery, Mod 33647, China) adjusting thickness to 0.75 mm. HUV were thawed progressively prior decellularization experiments.

*Agitation decellularization:* Agitation decellularization, or conventional method, was carried out using HUV segments (ID 5 mm, OD 6.5 mm x 85 mm). Samples were incubated in 100 ml of a solution composed of 20% acetone, 20% water and 60% ethanol. Bottles were placed on an orbital shaker (100 rpm) for 72h.

*Convective flow decellularization:* HUV segments of 85 mm length were placed in a perfusion bioreactor. A peristaltic pump was used to provide the solvent through the system (Cole Parmer L/S digital Standard Drive, model 07523-60) and pulse was diminished with a dampener. The pressure was recorded (PCL-818L. Advantech Co.,Ltd) and monitored on the computer screen (VisiDAQ, PCLS-920-31/P. Advantech Co.,Ltd.). Luminal pressure was maintained at 50±3 mmHg (CF-50 mmHg).
Figure 1. Convective flow method. A peristaltic pump drives the solution through the lumen side of the vessel at a constant flow rate of 50 ml/min. Pressure was measured downstream the vessel and monitored continuously with the data acquisition system.

Analysis of phospholipids and total protein extraction: samples of decellularization solution were evaporated (BUCHI rotavapor R-210/215) and then diluted in 5 ml of isopropanol. Phospholipids were measured with Phospholipids C (Wako Chemicals USA, Richmond, VA), a blue pigment was developed and absorbance measured at a wavelength of 600 nm. Total protein was determined with micro BCA protein Assay Reagent Kit (Pierce Biotechnology, Rockford, IL). Absorbance was measured at a wavelength of 550 nm.

Histological analysis: Samples were fixed in a 10% buffered formalin solution overnight, then dehydrated in graded ethanol (80%, 95%, 100% vol/vol) 15 min, 2x. Fixed tissue was embedded in paraffin, sectioned and mounted on glass slides. Following this, staining was completed using hematoxilin 7211 (Richard-Allan Scientific, Kalamazoo) and counterstaining with Eosin-Y (Richard-Allan Scientific, Kalamazoo). Stained sections were observed under light microscope.

Tensile test: testing was carried out at room temperature. Tissue specimens (n=9) were loaded onto the machine using stainless steel L-hooks. Stress was applied in circumferential direction, with an initial preload of 0.005 N at a rate of 5 mm/min and then elongated until failure. Stress was calculated as the applied force over area. Strain calculations considered the extension over original length of the sample. Value of the elastic modulus was obtained from the stress-strain graphs.

RESULTS

Biochemical assays

Phospholipids extraction profile: Quantitative analysis of decellularization solution showed that extraction of phospholipids decreased over time for the convective flow method and agitation. After three hours, the amount extracted with agitation was not significantly different from convective flow method. However, values obtained for 6 and 9 hours were significantly higher when convective flow was applied. The phospholipids extracted in the agitation experiments dropped considerably after 3h (from $176.0 \pm 30 \mu g$ to $15.4 \pm 1.7 \mu g$) and remained steady.
throughout decellularization. In contrast, convective flow extraction after three hours was 203.2 ± 37.7 μg and at six hours was 88.73 ± 9.8 μg.

Figure 2. Phospholipids extraction: Profile of extraction for convective flow and agitation methods tends to decrease over time. Error bars indicate standard error.

**Cumulative quantification of phospholipids:** In figure 3, extraction of phospholipids was represented as the mean cumulative amount over time. Total extraction of phospholipids was markedly greater with the convective flow than agitation. The rate of extraction during the first three hours was 67.7 ± 12.6 μg/hr for convective flow, whereas agitation gave a mean of 58.7 ± 10 μg/hr. After 72 hours, the total amount of removed phospholipids was 485.89 ± 20.19 μg for convective flow, while agitation led to a total of 214.56 ± 14.63 μg.

Figure 3. Cumulative phospholipids: amount of extracted phospholipids increases during the first 12 h with convective flow method. After 12 h, extraction tends to plateau.
**Proteins extraction profile.** The rate of protein extraction seemed to decrease over time for agitation and convective flow methods (figure 4). After 3h convective flow had extracted $5.6 \pm 0.23$ mg of soluble protein whereas agitation extracted $1.3 \pm 0.35$ mg. 50% of the total amount extracted with convective flow had been removed during the first three hours of the experiment. Between 3 and 6 h, the amount extracted corresponded to 28% of the total when using convective flow and 8.5% when agitated.

![Figure 4. Soluble protein extraction](image)

**Figure 4. Soluble protein extraction.** Soluble proteins decreased over time for convective flow. Extraction after 3h corresponds to 50% of net protein extracted after 72 hours.

**Cumulative quantification of total protein:** Average values of removed soluble proteins were added up and represented in figure 5. The cumulative extraction of total proteins for CF-50 was approximately linear in the first 9 h ($r^2=0.97$). Convective flow protein rate of extraction was 1.1 mg/h while agitation extraction rate was 0.09 mg/h. Total protein removed with convective flow after 72 h was 3.5 times greater than with agitation; $11.8 \pm 0.6$ compared to $3.1 \pm 0.3$ mg.
Figure 5. Cumulative total protein: cumulative extracted protein remained higher with convective flow method than with the conventional agitation method. After 12 h the amounts extracted with both methods reached a plateau.

Mechanical properties: As shown in figure 6, the stress-strain relationship characteristic for connective tissue was preserved after decellularization (agitation and convective flow). The mechanical properties of the acellular HUV were compared with those of the non treated tissue (cellular). Modulus of elasticity for acellular tissues (agitation decellularization and convective flow methods) was not statistically different from cellular controls (2.43 MPa and 2.82 MPa compared to 2.44 MPa respectively).

Figure 6. Representative Stress-Strain Analysis. The biphasic stress-strain relationship seen in native blood vessels was retained. Characteristic “toe region” was observed for both decellularization methods followed by a linear region used from where elastic modulus was calculated.
**Histological analysis:** The images obtained from histological analysis demonstrated that the circumferential collagen fibers present in native HUV were preserved even after agitation and convective flow decellularization methods. Images of the agitation decellularized samples revealed the presence of cells. In contrast, samples that underwent convective flow are void of cells in almost the overall area.

**Figure 7. Light micrographs of treated HUV.** Hematoxylin and eosin stained samples revealed the presence of cells after agitation decellularization (A). Cells were not present after convective flow decellularization (B). Loosening of the fibers was detected.

**DISCUSSION**

The main goal of decellularization treatments is the removal of the major immunologic components like lipids, soluble proteins and cellular components while preserving the major extracellular proteins such as collagen and elastin fibers [6]. Extracellular matrix components are greatly conserved and well accepted among species and additionally retain their natural shape even after treatment [2]. Conservation of the biomechanical properties of the tissue is relevant in the overall outcome of a decellularization process. Treatments must efficiently remove cellular components while preserving mechanical properties and structure nearly identical to those in native state. Decellularization treatments are commonly based on chemical agents coupled to enzymes. Although these treatments have had promising results, difficulty arises in the case of more complex structures like blood vessels or heart valves [4, 9]. In other cases to achieve a complete cell removal higher concentrations of chemicals were required [10]. In addition, these chemical agents can potentially damage the extracellular matrix or remove desired components like glycosaminoglicans [1].

The overall goal of this research was to assess the convective flow method as an alternative treatment for the preparation of acellular ex-vivo matrices suitable for tissue engineering applications. Our model introduces the use of a bioreactor where chemical agents are used along with physical forces to decellularize human umbilical veins (HUV). The pressure gradient between the lumen and alburnen side of the HUV produces a convectional flow through the cross section of the sample than enhances cell elements removal while maintaining structural integrity.
Assessment of phospholipids is greatly important since remaining lipids in ex-vivo matrices may promote undesired effects like calcification. After specific removal of lipids with either chloroform/methanol or sodium dodecyl sulfate (SDS) studies showed a reduced calcification of the tissue in a rat model [11]. Convective flow method demonstrated to be more efficient in the removal of lipids than the agitation method. After 6 hours of experiment completion, the convective flow removed 291 ± 17 μg. This value was 1.3 times higher than the total amount removed with agitation upon experiment termination (72 hours).

Moreover, remaining soluble proteins or cellular debris elicits host immune response. Therefore, removal of all cellular components or soluble proteins plays an important role in the success of an implanted graft. Acellular vascular conduits that were produced after detergent and enzymatic extractions, showed no inflammation after implantation in dogs of a different breed [12].

Decellularization with convective flow resulted in a more efficient removal of soluble proteins from the HUV than the agitation. Only after the first three hours we observed that the amount removed was 4.2 times greater than when samples were decellularized under agitation. The mechanism of action of the convective flow provides a more uniform solvent delivery throughout the vein wall. Furthermore, the applied pressure acts as a mechanical force to burst the cells while the convective flow washes out the cell debris across the vein wall.

Examination of the extracellular matrix revealed that the collagen structure of the human umbilical vein was conserved after agitation and convective flow. Using convective flow, scaffolds were void of endothelial cell lining and of the smooth muscle cells in the tunica media. However, the collagen fibers after convective flow treatment appeared to be loosely packed when compared to the samples that were agitated. The preservation of the natural structure of the scaffold is highly important to provide a natural environment onto cells can adhere and proliferate and differentiate [2].

Adequate strength to stand the physiological forces is a requirement for any tissue graft. Results showed that the mechanical properties of the decellularized HUV remained remarkably similar to those of native tissue. For instance, the applied pressure during the convective flow decellularization had not a negative effect in the extracellular matrix. The modulus of elasticity and maximum strength were statistically similar to those of fresh tissue. More importantly the conservation of the cylindrical shape of the vein was favored when convective flow was applied.

Collectively, the findings suggested that the convective approach was successful at decellularizing complex structures. The potential of the convective flow as a mechanism of tissue decellularization was assessed and its superior abilities over traditional agitation methods were demonstrated. This method is being investigated to develop a multi-step process to decellularize, then culture constructs within the same bioreactor, to create engineered vascular tissues while minimizing graft handling upon clinical implantation.
REFERENCES

Development of a Human Umbilical Vein-derived Periodontal Grafting Matrix

Selda Goktas, Nicolas Pierre, and Peter S. McFetridge
University of Oklahoma, Norman, OK 73019

ABSTRACT

Our approach in this study was to develop a novel Human Umbilical Vein (HUV)-derived 3D ex-vivo scaffold and investigate its potential as an alternative grafting material for the diseased soft connective tissue in periodontal tissue engineering. The HUV was prepared by a decellularization process to render the ex vivo material immunologically inert. Uniaxial tensile test, stress relaxation and suture retention tests were performed to evaluate the biomechanical response of the material as a periodontal graft. The biomechanical response of HUV was found to be greatly dependent upon the composition of its extracellular components and the arrangement of the fibrous proteins (collagen, elastin, glycoprotein microfibrils). Morphological analysis of the seeded HUV sections assessed with scanning electron microscopy (SEM) demonstrated the human gingival fibroblasts (hGFs) have the tendency to adhere on HUV biosurface. The cellular studies showed the in vitro proliferation rate of hGFs were significantly different on lumenal and ablumenal surfaces of HUV due to their distinct biological and architectural properties. Our results have shown HUV bioscaffold represents a promising natural derived surgical barrier for periodontal tissue engineering.

1. INTRODUCTION

Periodontal disease is a chronic mixed bacterial infection leading to a progressive loss of bone and soft tissue support of the teeth. For many years, the underlying goal of periodontal therapy has been to regenerate tooth support that has been destroyed by periodontal diseases. This involves a complicated and coordinated sequence of wound healing events resulting in the formation of new cementum, periodontal ligament, and alveolar bone in association with a previously diseased root surface. The feasibility of periodontal guided tissue regeneration (GTR) was demonstrated in a proof of principle study in 1982 [1], and employed a physical barrier interposed between the periodontal defect and overlying soft tissues. The concept was that the barrier provided a protected wound healing environment that excluded gingival epithelial and connective tissue cells and allowed repopulation of the wound space by undifferentiated mesenchymal cells from the remaining healthy periodontal ligament and alveolar bone.

In this study, we are proposing the use of human umbilical vein (HUV) as an alternative natural, collagenous surgical barrier for the regeneration of oral tissues in a variety of clinical situations. The HUV is allogenic which reduces immune reactivity and the risk of interspecies and the risk of interspecies viral transfer. Its vascular derivation improves compliance, is conducive to cellular attachment and subsequent remodeling [2-5]. Moreover, HUV is bioabsorbable and there is no need for a second surgery to retrieve the material.
Due to structural and morphological variation between the ablumenal and lumenal surfaces of the *ex vivo* acellular HUV material, we expect discrete cellular and biomechanical responses from each surface of the biomembrane. Our vision for dental reconstructive surgery is that the highly porous ablumenal surface composed of type I collagen and hyaluronic acid will be implanted against the wound interface, with the minimally porous, smooth, type IV collagen luminal surface at the oral cavity/gingival interface (Figure 1). The tissue-engineered HUV would be surgically implanted over the wound bed, under the residual healthy gingiva.

![Figure 1. A diagram of the proposed surgical application for a periodontal defect, where the HUV matrix is inserted under the existing gingiva.](image)

In order to investigate the biomechanical properties of our human umbilical vein (HUV)-derived composite material, we subjected the scaffold to some mechanical tests to predict its response to the forces induced in physiological oral environment. Besides, cellular studies were conducted to assess the ability of the 3D-*ex vivo* HUV scaffold to provide a suitable environment for the attachment, growth and proliferation of hGF.

2. MATERIALS & METHODS

2.1 Preparation of Human Umbilical Vein (HUV)

Human umbilical cords were obtained from the Delivery Suite of Norman Regional Hospital (Norman, OK) and washed with double-distilled water. The cords were mounted on stainless steel mandrels and placed in a polystyrene canister to allow progressive freezing (2.5°C/min) of the cords to -80°C for at least 48 h [6]. The vessels were removed from the freezer and immediately fixed between the headstock and the tailstock of the lathe. Human umbilical vein (HUV) segments were dissected to wall thicknesses of 400 and 800 μm by rotary lathing under computer control. Each tissue section was put in -20°C immediately after dissection and stored for 1-2 h. In order to thaw the sections progressively, the cords were then placed in +5°C and kept for and kept for no more than 24 h. Vessels were taken out of the freezer and allowed to thaw at room temperature for 1h prior to decellularization.
2.2 Decellularization of HUV Tissue Sections
The thawed HUV’s were cut longitudinally to generate flat sheets and placed in glass bottles containing sterilized sodium dodecyl sulphate (SDS) (Mallinckrodt Baker, Inc., Phillipsburg, NJ) solution and agitated on an orbital shaker (100 rpm, 24 h, room temperature). The decellularization solution was decanted and the tissue samples were washed in excess amount of distilled water for several washes to remove all the residual surfactant. The samples were then incubated overnight at room temperature in phosphate buffered saline (PBS, Gibco Life Technologies, Grand Island, NY) containing 70 U/mL DNase I (Sigma-Aldrich Inc., St. Louis, MO) to digest the DNA. Then, the tissue sheets were thoroughly washed using PBS by replacing the washing solution several times to remove any residual DNase. Samples were then terminally sterilized using 0.2% peracetic acid/ 4% ethanol solution for 2h. Traces of peracetic acid were removed and the pH was returned to approximately 7.4 by rinsing the tissue samples in PBS.

2.3 Uniaxial Tensile Testing
A uniaxial tensile testing machine (Instron Corporation, Model 5542, Norwood, MA) was used for the stress-strain analysis of HUV sections. Tissue strips (3 mm wide×18 mm long) with longitudinal and circumferential orientations were cut from the decellularized HUV sheets with a scalpel blade cutter. The tissue strips were preloaded to 0.003 N at a cross-head velocity of 5 mm/min and then preconditioned with five repeated cycles of loading up to a fixed load and then unloading at the same extension rate, 5 mm/min [7-10]. Finally, the strips were stretched to failure at an extension rate of 5 mm/min. The load and displacement data were recorded and tensile properties of the HUV tissue strips were deduced from these records.

2.4 Stress Relaxation Test
The viscoelastic tissue response of HUV was captured by performing stress relaxation test. The tissue strips were preloaded to 0.003 N, and then preconditioned in five repeated cycles. The cross-head velocity was 5 mm/min for preloading and preconditioning, the same value used for the uniaxial tensile test. The samples were then loaded to one-third of the force at fracture at an extension rate of 30 mm/min and then the cross-head was stopped immediately to unload the sample [11]. The initial stress, \(\sigma_0\) at \(t=0\) and the equilibrium stress, \(\sigma_{\infty}\) at \(t=180\) s were recorded after the relaxation was initiated. The contribution of the viscoelastic stress to the total stress, \((\sigma_0-\sigma_{\infty})/\sigma_0\) was evaluated for each sample [12].

2.5 Suture Holding Capacity
Suture holding capacity experiment was performed on the sutured tissue sheets (9 mm wide×18 mm long) applying uniaxial stress (Instron Corporation, Model 5542, Norwood, MA). A single sterile 3-0 braided silk suture was passed through one end of the tissue section 3 mm below the edge, with the other glued to sand paper and then attached to the grip. Samples were preloaded and preconditioned the same as in the uniaxial tensile testing, and then stretched until failure at an extension rate of 5 mm/min.

2.6 Cell Culture
Human gingival fibroblasts (hGF) were obtained from a patient and cultured under standard cell culture conditions within a humidified incubator at 37°C with 5% CO2. Cells were maintained
with Dulbecco’s Modified Eagle’s Medium (DMEM) supplemented with L-glutamine, 100 U/ml penicillin, 100μg/ml streptomycin (Gibco Life Technologies, Grand Island, NY), and 10% animal serum complex (FetalPlex™, Gemini Bio-Products, West Sacramento, CA). The medium was changed every 2 days.

2.7 Quantification of DNA Content within the Scaffold
Cells were released by digesting each disc in 1 ml of 200 U/ml DNA-free, sterile-filtered collagenase solution (Sigma-Aldrich Inc., St. Louis, MO) in PBS. The discs were incubated for 2 hours at 37°C till complete digestion. The released cells were lysed by three freeze thaw cycles at -80°C to liberate the DNA. Samples were then incubated with 1 mL of PicoGreen reactant for 5 minutes at room temperature protected from light. Fluorescence was measured at excitation and emission wavelengths of 485 and 535 nm, respectively.

2.8 SEM Surface Analysis
Tissue sheets (3 mmx7 mm) were fixed in 1 % (v/v) glutaraldehyde (Sigma, St. Louis, MO), treated with 1% (v/v) osmium, then gradually dehydrated with ethanol solutions. This was followed by the critical point drying (Autosamdr-814, Tousimis, Rockville, Maryland) and gold sputtering (Hummer IV). The tissue sections were treated with PBS to remove any residual chemical after each treatment. Finally, surface images were taken using a JEOL LSM-880 Scanning Electron Microscopy (SEM).

2.9 Statistics
Nine different specimens from each group were tested for the mechanical tests and for the cellular study, data were collected in triplicates at time point for the ablumenal and luminal surfaces. Analysis of variance (ANOVA) with Tukey HSD test was performed to evaluate the statistical significance of the differences between results. The difference was considered statistically significant when \( p<0.05 \).

3. RESULTS

3.1 Uniaxial tensile testing
The tensile properties of the HUV strips were deduced from the uniaxial tensile test data and the mean values for the Young’s modulus, ultimate tensile strength, failure strain and failure load of the tested samples were presented in Table 1.

<table>
<thead>
<tr>
<th>Material</th>
<th>Cutting Thickness (mm)</th>
<th>Young’s Modulus (N/mm²)</th>
<th>Ultimate tensile strength (N/mm²)</th>
<th>Tensile strain at failure</th>
<th>Tensile load at failure (N)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HUV longitudinal</td>
<td>0.4</td>
<td>15.4 ± 1.38</td>
<td>1.44 ± 0.13</td>
<td>0.30 ± 0.04</td>
<td>3.77 ± 0.31</td>
</tr>
<tr>
<td></td>
<td>0.8</td>
<td>7.40 ± 3.55</td>
<td>0.69 ± 0.07</td>
<td>0.26 ± 0.07</td>
<td>3.98 ± 0.37</td>
</tr>
<tr>
<td>HUV circumferential</td>
<td>0.4</td>
<td>6.06 ± 1.16</td>
<td>0.60 ± 0.18</td>
<td>0.24 ± 0.02</td>
<td>1.58 ± 0.46</td>
</tr>
<tr>
<td></td>
<td>0.8</td>
<td>3.15 ± 0.72</td>
<td>0.38 ± 0.03</td>
<td>0.28 ± 0.05</td>
<td>2.19 ± 0.19</td>
</tr>
</tbody>
</table>
3.2 Stress Relaxation Test
The parameters deduced from the stress-relaxation data were reported in Table 2. The amount of relaxation was greater for the longitudinal strips suggesting the contribution of the stress of the viscous components to the total stress was larger for the longitudinal strips. Furthermore, the relaxation response of the tissue indicated a variation in the composition of the viscoelastic components through the HUV wall.

Table 2. Results of stress-relaxation tests

<table>
<thead>
<tr>
<th>Material</th>
<th>Cutting thickness (mm)</th>
<th>$\varepsilon_0$</th>
<th>$\sigma_0$ (N/mm$^2$)</th>
<th>$\sigma_\infty$ (N/mm$^2$)</th>
<th>$(\sigma_0-\sigma_\infty)/\sigma_0$</th>
</tr>
</thead>
<tbody>
<tr>
<td>HUV longitudinal</td>
<td>0.4</td>
<td>0.09±0.01</td>
<td>0.16±0.01</td>
<td>0.06±0.02</td>
<td>0.65±0.13</td>
</tr>
<tr>
<td></td>
<td>0.8</td>
<td>0.12±0.01</td>
<td>0.14±0.02</td>
<td>0.07±0.02</td>
<td>0.52±0.11</td>
</tr>
<tr>
<td>HUV circumferential</td>
<td>0.4</td>
<td>0.11±0.02</td>
<td>0.16±0.01</td>
<td>0.08±0.01</td>
<td>0.50±0.09</td>
</tr>
<tr>
<td></td>
<td>0.8</td>
<td>0.16±0.03</td>
<td>0.14±0.01</td>
<td>0.08±0.01</td>
<td>0.41±0.05</td>
</tr>
</tbody>
</table>

3.3 Suture Holding Capacity
The suture holding capacity of the 800 µm-thick, circumferential HUV strips (2.15 ± 0.27) was significantly higher than the longitudinal strips for the same thickness (1.62 ± 0.17). However, there was no significant difference in the failure rates of the strips cut in longitudinal (1.38 ± 0.21) and circumferential (1.52 ± 0.27) directions for 400 µm cutting thickness.

3.4 Cell Attachment and Growth in Scaffolds
Figure 2A shows the proliferation rate of hGFS seeded on tissue disks over time assessed with Picogreen assay. Cell density was found significantly greater for the ablumenal (77097 ± 2836.6) surface than the lumenal surface (41800 ± 4175.2). The SEM images revealed the hGFS have a high tendency to attach on the ablumenal (Figure 2B) and lumenal (Figure 2C) surfaces of the acellular HUV scaffold.
Figure 2. Picogreen assay and SEM analysis conducted on discs seeded with hGFs. The cell density was found to be significantly higher for the ablumenal surface reflecting a higher cell proliferation on this surface (A). The SEM pictures show human gingival fibroblast adhering to the ablumenal (B) and lumenal (C) surfaces of HUV.

4. DISCUSSION

When human umbilical vein (HUV) is susceptible to uniaxial tension, first the elastin fibers start to align in the loading axis. Since the applied tensile force required to stretch the elastin fibers is not enough to initiate the recruitment of the collagen fibers, collagen fibers need greater force to initiate their realignment in the stretching axis. The wavy architecture of collagen fibers which is observed most at the zero-load state gradually decreases as the tissue is strained uniaxially and the fibers become more aligned in the direction of stretching axis. Strain-induced parallel realignment of the collagen fibers in the direction of loading caused a progressive increase in the stiffness of the tissue and hence, allowed the collagenous specimens to bear further load before failure [13].

As far as the uniaxial tensile characteristics of the tissue strips in different orientations was concerned, HUV showed anisotropic response with its longitudinal and circumferential strips having different stress-strain behaviors. The anisotropic nature of the material was attributed to the variation in orientation of the fibers transmitting the tensile load within the 3D matrix, hence contributing to the strength of the material. The greater tensile strength of the longitudinally-oriented samples can be attributed to the possibility that most of the medial collagen fibers are aligned along the axial direction of HUV rendering the orthotropic mechanical behavior of the material, which will show higher stiffness for the tissue sections oriented parallel to the fiber direction in order to resist uniaxial tensile load [14, 15]. Also, medial collagen fibers predominantly bear load in the axial direction, therefore the longitudinal strips could resist more uniaxial tensile load.

On the other hand, most of the collagen fibers in circumferential strips were assumed to be oriented initially perpendicular to the loading axis, and as the stretching of these strips progressed, the fibers started to realign themselves parallel to the loading axis. Unfortunately, the
circumferential strips fracture before all of its collagen fibers had enough time to reorient themselves in the stretching axis and contribute to the strength of the material. Since the extent to which the tissue can bear the applied uniaxial tension depends primarily on the amount of fibers aligned in the axial direction to transmit and carry the load [16, 17], the cross-loaded circumferential strips demonstrated lower mechanical strength after a complex realignment process [18]. Our approach on the possible orientation of medial collagen fibers was also supported with the suture retention tests.

The uniaxial tensile testing of the HUV strips with varying wall thickness demonstrated stress-strain behavior gradients through the wall thickness of HUV. This indicated the heterogeneity of the material in terms of the composition of the extracellular components, density of the fiber packing and the diameter of the collagen fibrils. The tissue layer consisting of densely-arranged fibrous connective tissue showed higher tensile strength, whereas the layer having relatively more loose connective tissue could retard smaller loads. In summary, the medial layer exhibited higher uniaxial tensile strength and anisotropy with its unidirectional collagen fibers mostly aligned in the axial direction, whereas the tissue layer surrounding the media indicated lower mechanical strength with randomly organized fibers.

The viscoelastic nature of HUV was demonstrated by the stress-relaxation test, in which the stress on the strained tissue at a fixed extension decreases rapidly at first and gradually decays until the stress value reaches equilibrium [17]. Internal rearrangement of collagen network morphology could be driven by the initial strain energy imparted to the specimen on stretching, which gradually diminishes as the stress relaxes [13]. The greater the difference between the initial and the equilibrium stress values, the lesser degree the tissue deforms and higher the capacity of the tissue to recover its initial fiber alignment. Also, the tissue section showing relatively more rapid decay in force was pronounced to be more viscoelastic.

The HUV strips consisting relatively higher amount of Wharton's jelly indicated lower elasticity, whereas the sections consisting primarily of the medial layer demonstrated greater viscoelastic behaviour. This result can be attributed to the fact that the medial collagen fibers and the smooth muscle components were the primary constituents influencing the viscoelasticity of the material. Due to the resemblance of the uniaxial tensile and viscoelastic properties of HUV, it is most likely that the desired orientation of the collagen fibers and the composition and organization of the ECM components influencing the tensile strength of the material also had a profound effect on the relaxation processes of HUV.

Further comparison was made on ability of human gingival fibroblasts (hGFs) to adhere, grow and proliferate on the HUV biomatrix under static growth conditions. Cells appeared to adhere on both surfaces of the scaffold, and the proliferation rate assessments of hGFs indicated the cells had greater tendency to proliferate on the ablumenal surface of HUV. This result can be attributed to the more densely packed morphology of the extracellular matrix (ECM) components including the fibrous proteins on the luminal surface inhibiting further transport of the adhered cells into the matrix. The higher porous structure enhancing the migration of hGFs through the tissue could be another reason, but the quantitative analysis of the porosity of the HUV-derived scaffold remains to be analysed.
In accordance with the promising results, we believe a construct incorporating the HUV bioscaffold has the necessary physical and biological properties to serve as a strong, elastic tissue patch that will enable dental surgeons to rebuild oral soft tissues without requiring autologous tissue transplants. We are now expecting the methods used to create an HUV-derived ECM scaffold to significantly alter the biomechanical and biological responses of the material and for our further research, we are to determine the influence of processing conditions on the material properties of HUV. We also aim to further our understanding of this novel graft by quantifying the regenerative capacity of the scaffold under defined environmental and mechanical conditions that mimic the oral wound environment.

ACKNOWLEDGEMENTS
Oklahoma Center of Advancement of Science & Technology (OCAST) (HR05-157) primarily supported this work. Authors would also like to acknowledge the Norman Regional Hospital (Norman, OK) for providing the human umbilical cords used in this study.

REFERENCES


Flow in Renal Artery Aneurysms and Hypertension

Linden Heflin, Edgar O'Rear, Dimitrios Papavassiliou, Carrie Street

School of Chemical, Biological and Materials Engineering
University of Oklahoma, Norman, OK 73019

Abstract

One cause of hypertension is a malfunctioning in the renin-angiotensin-aldosterone system (RAAS). The RAAS is a renal hormonal control system that regulates systemic blood pressure by sensing the blood pressure inside the kidney and releasing a vasoconstrictor to correct low blood pressure. The malfunction of the RAAS associated with hypertension could then be induced by a large pressure drop caused by abnormal structure of the renal artery, fooling the RAAS into lowering systemic blood pressure. Using computational fluid dynamics it is possible to model flow for various renal artery geometries. This research seeks to evaluate common aneurysm geometries occurring in the renal artery as a cause of hypertension. The pressure difference for typical renal arteries has been determined as a baseline for comparison. The effect of aneurysm size, location, and placement relative to the artery has been investigated in silico. Results show that the pressure difference in the presence of an aneurysm only is not significantly higher than that for a typical renal artery. These findings have led to new hypotheses of how aneurysms could increase the pressure difference across the renal artery.

Introduction

The kidney constitutes a major organ whose main function is to filter the body's blood. Less well appreciated is its involvement in the regulation of blood pressure. This regulation is achieved through the use of a feedback control loop called the Renin-Angiotensin-Aldosterone System (RAAS). Baroreceptors located in the afferent arterioles of the kidney sense the blood pressure. If this blood pressure registers as too low, the kidney releases renin, initiating the RAAS. The hormones released during this step of the feedback loop cause vasoconstriction throughout the body, which raises the systemic blood pressure.

In 1934, Goldblatt induced persistent renin-dependent hypertension by placing clamps to constrict the renal arteries of canines\(^1\). By doing this, it was demonstrated that alterations in renal artery geometry can induce hypertension. This research is the motivation for the current project, in which multiple alterations to renal artery geometry are studied.

The hypothesis behind this project is related to the effect of abnormal renal artery geometries on apparent blood pressure and the RAAS. The RAAS would be expected to respond to aberrant renal artery geometries, which would cause a larger than normal pressure drop across the renal artery, by releasing renin and increasing the blood pressure in the body.

A renal artery stenosis is a pathologically occurring narrowing of diameter similar to that employed by Goldblatt\(^1\). Stenoses alter the local hemodynamics. Patients that exhibit renal
artery stenoses often present with hypertension. However, this may not be the only renal artery abnormality that can lead to renin-dependent hypertension. Renal artery aneurysms (RAA) are another alteration in renal artery geometry that may lead to hypertension. Recently, it has been shown that the instance of renal artery aneurysms is higher than previously estimated. Moreover, 70% of patients presenting with renal artery aneurysms have hypertension. Investigating the pressure drop through a renal artery with an aneurysm will indicate whether or not this etiology is related to a significant pressure drop caused by the aneurysm. Additionally, critical renal artery aneurysm sizes and locations can be determined in order to better predict renin-dependent hypertension due to renal artery aneurysms.

Methods

Computational Fluid Dynamics (CFD) is a way in which the hemodynamics of the renal artery system can be studied. Using CFD, various 2D models of different renal artery geometries can be created. CFD models the behavior of flow fields by numerically solving equations representative of the system. In contrast to difficult in vivo pressure measurements, CFD offers a promising means for investigating renal artery structural pathologies and corresponding pressure drop characteristics.

Simulations of blood flow through the renal artery were carried out using the flow simulation program Fluent and the preprocessing program Gambit. Gambit version 2.2.30 served as the graphical user interface for creating the representative renal artery geometries and their respective computational meshes or grids. The calculation used to determine the pressure difference through the renal artery was simply the difference between the average pressure at the outlet and the average pressure at the inlet of the computational domain. The simulation conditions are given below.

**Simulation Conditions**

- Steady flow
- Laminar model
- Fluid properties
  - $\mu = 4$ cp
  - $\rho = 1060$ kg/m$^3$
- Boundary Conditions
  - Inlet: Constant Velocity
  - Outlets: Constant Pressure Outlets
- Flow Conditions
  - $Q = 600$ ml/min
  - $Re = 674$
- Dimensions
  - Renal artery length = 50 mm
  - Renal artery diameter = 5 mm
  - Diameter of daughter arteries = 3 mm
An image indicating how the 2D model for this project was developed is given in Figure 1.

![Image of 2D model of renal artery](image)

**Figure 1:** 2D model of renal artery, main branch, 2nd and 3rd generation branches (right panel). Panel on the left is from Porcoro, et al.

**Results**

In order to establish a baseline pressure difference for a typical renal artery, simulations were performed using a typical renal artery model. Figure 2 shows the contours of velocity magnitude for this typical renal artery. The pressure difference found for this geometry was 0.9 mm Hg.

![Image of contours of velocity magnitude](image)

**Figure 2:** Contours of Velocity Magnitude for Typical Renal Artery
In order to begin looking at the effect of renal artery aneurysms on pressure difference across the renal artery, the most common aneurysm location was determined from the medical literature. According to Henke et al\textsuperscript{2}, the most common single location for a renal artery aneurysm is at the first bifurcation of the renal artery.

Five different RAA radii were studied at the main branch in order to determine the significance of aneurysm presence and the dependence of pressure difference on aneurysm radius. Figure 3 shows the contours of velocity magnitude for a renal artery aneurysm with a radius of 6.5 mm. Figure 4 shows how pressure difference varies with RAA radius. The pressure difference increases with RAA radius up to the largest radius. The drop off in pressure difference for this largest radius is probably due to the loss of a second generation branch because of how large the aneurysm is. Although pressure difference generally increases for all of the cases compared to the pressure difference for a typical renal artery, the difference is not considered significant.

**Figure 3:** Contours of velocity magnitude for a RAA with radius of 6.5 mm
Simulations were also performed for asymmetric RAA of the main branch. The asymmetry was modeled by a protuberance out of the main bifurcation. Figure 5 shows geometry of the asymmetric aneurysm simulations and the contours of velocity magnitude.

Figure 5: Contours of velocity magnitude for asymmetric RAA, radius = 6.5 mm

Figure 6 shows how the pressure drop through the renal artery varies with aneurysm radius for the asymmetric RAA. This geometry also did not show a significant difference compared to the pressure difference for a typical renal artery.
These results indicate that an aneurysm of the main renal artery bifurcation does not have a significant impact on the pressure difference across the renal artery. Two other aneurysm locations were studied in order to determine if aneurysm location has a noteworthy effect on the pressure difference. Figure 7 shows the 2D models of these geometries.

The pressure drops for these different aneurysm locations were not significant compared to that for the typical renal artery.

All of the previous renal artery aneurysm locations did not exhibit a significant contribution to the pressure difference through the renal artery. However, as previously mentioned, 70% of RAA patients also exhibit hypertension. Because of this association, a case study in which a patient had a large RAA of know geometry as well as severe hypertension was modeled. In this case, the patient had a large saccular renal artery aneurysm of the main renal artery. The aneurysm was repaired, and the patient’s hypertension was greatly lessened. Figure 8 shows an image of the patient’s aneurysm as well as the contours of velocity magnitude from the simulation.
The results of this simulation showed that simply the presence of the saccular renal artery aneurysm did not affect the pressure difference across the renal artery. As can be seen from the velocity contours for this simulation, the recirculation into the aneurysm is minimal, and does not seem to affect the velocity in the artery.

Because of these results, it can be hypothesized that some other phenomena associated with the aneurysm may be causing a large pressure difference. This could be caused by a blockage occurring due to the weakened artery wall around the aneurysm as depicted in Figure 9.

Figure 9: Contours of velocity magnitude for RAA with associated blockage

Figure 10 shows the pressure difference versus constriction diameter for various blockage geometries. This plot shows that the pressure difference decreases with increasing constriction diameter, which is expected from classical fluid dynamics. For the most severe constriction
diameters, the increased pressure difference is very significant compared to that for a typical renal artery. However, this influence on pressure difference may not be due to the aneurysm and may solely be caused by the blockage. The simulations were performed again with the same constriction diameters, but without the aneurysm. Figure 11 shows the pressure difference for the simulations performed with and without the aneurysms present. As can be seen from this plot, the pressure difference with and without the aneurysm is not significantly different. This indicates that the influence on pressure difference is due to the hypothetical blockage and not the aneurysm.

Figure 10: $\Delta P$ versus constriction diameter for aneurysm + constriction

Figure 11: $\Delta P$ versus constriction diameter for constriction associated with aneurysm and for constriction only
Conclusions

Through this project, aneurysms have been successfully modeled using computational fluid dynamics. From the simulations, it can be observed that the pressure difference across the renal artery increases with increasing aneurysm radius, however the difference is not significant when compared to a typical renal artery. Because of these results, another factor must be involved in inducing hypertension for aneurysmatic patients. One factor that may be involved is a blockage or constriction associated with the aneurysm.

Future Work

Future work for this project would be to investigate other abnormal renal artery geometries that could be inducing hypertension. This would lead to the development of guidelines for renal artery abnormalities of concern. Additionally, the incorporation of oscillatory flow and fluid-structure interactions between the blood and the artery wall will increase the accuracy of the simulations. The results of these types of simulations may indicate that the aneurysm lends a larger contribution to the pressure difference than the results of this study indicate.

References

Fiber Optic Oxygen-Based Biosensors for Measurement of Toluene in Groundwater

Zhong Zhong¹, David S. Dandy¹, Sean B. Pieper², Kevin L. Lear², Thomas K. Wood³, Kenneth F. Reardon¹

¹Department of Chemical and Biological Engineering, Colorado State University, Fort Collins, Colorado 80523-1370, ²Department of Electrical and Computer Engineering, Colorado State University, Fort Collins, Colorado 80523-1373, ³Department of Chemical Engineering, Texas A&M University, College Station, TX 77843-3122

Abstract

Measurements of groundwater contaminants such as toluene are critical to site characterization and bioremediation monitoring. Fiber optic oxygen-based biosensors have great potential to provide cost-effective, real time, in situ measurements. These biosensors are made by using oxygenase-expressing bacteria as the biocomponent and oxygen optodes as the transducer. Initial experiments have demonstrated that the response of the oxygen optodes is $\Delta V/\Delta C_{O_2} = 34.5 \text{ mV-L/mg (O}_2\text{)}$ and that steady signal was obtained within = 1 minute of a decrease in oxygen concentration. The signals of oxygen-based biosensors increased after additions of toluene to aqueous solutions with a sensitivity of $\Delta V/\Delta C_{\text{toluene}} = 0.214 \text{ mV-L/mg (toluene)}$. However, the time to achieve a steady signal was significantly longer for the biosensors than for the oxygen optodes due to the limitation of diffusion in the bacterial sensing layer.
Monte Carlo Simulation of Photoelectrochemical Disinfection of Bacteria

A. Argoti and L. T. Fan
Department of Chemical Engineering, Kansas State University, 1005 Durland Hall, Manhattan, KS 66506, U. S. A.

Abstract
Currently, the thorough disinfection of pathogens in general and bacteria in particular is of keen public interest in view of various medical needs, public health concerns, and food safety and security. The populations of bacteria comprise discrete and mesoscopic organisms, thereby giving rise to random or stochastic fluctuations in the macroscopic variables describing them, e.g., their number concentration. Thus, it is desirable that the analysis, modeling and simulation of the disinfection of bacteria be carried out in light of a stochastic paradigm, which not only estimates the process’ mean or average value but also quantifies the process’ fluctuations in terms of higher moments about the mean, e.g., variance. Nevertheless, the governing equation of a stochastic model can be exceedingly difficult to solve, especially if it is non-linear; such complexity can be circumvented by Monte Carlo simulation. Herein, the non-linear governing equation of a stochastic model for the photoelectrochemical disinfection of E. coli is simulated by the Monte Carlo method via the event-driven and time-driven approaches. The resultant mean and variance from simulation are compared with the available experimental data as well as with their corresponding analytical solutions.

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Sulfate reducing microbial diversity in the sediments of Lake Coeur D’Alene

Isha Chhatwal, Rajesh K. Sani, Brent Peyton¹, Timothy Ginn², and Nicolas Spycher³

South Dakota School of Mines and Technology
¹Montana State University, Bozeman, MT,
²University of California, Davis, CA,
³Lawrence Berkeley National Laboratory, Berkley, CA

ABSTRACT

High concentrations of heavy metals in oceans, lakes, and rivers have rapidly become a global problem in water supply and related ecosystems. Increase in the metal contamination results of historical mining in upstream in Lake Coeur d’Alene (LCA) has lead to study of interactions of heavy metals with indigenous microorganisms. Microorganisms present in heavy metals (including Pb, Cu,Zn)-contaminated lake sediments show the presence of sulfate reducing activity which can precipitated metals as metal sulfides which is an attractive alternative strategy for remediation of metal-contaminated subsurface environments. Lake sediment samples were enriched for sulfate reducing bacteria (SRB). To find out the total SRB diversity, total DNA was extracted, and amplified for 16S rDNA and DSR (disulfite reductase) gene using polymerase chain reaction (PCR) technique. Two clone libraries were made: i) 16S rDNA mixed SRB cultures, ii) DSR mixed SRB cultures. In addition, DNA of Desulfovibrio desulfuricans G20 was also extracted and amplified for 16S rDNA and DSR and cloned. Sequencing is being carried out. This will give us info about the SRB diversity present in the metal-contaminated sediments of lake. Future work is to identify the selected pure isolates, evaluate Pb, Cu, and Zn inhibition kinetics, develop mathematical models to predict metal inhibition in metal-contaminated environments including LCDA.
Evaluation of Tetrachloroethene (PCE) Degradation in Contaminated Ground Water

J. H. Ibbini¹, L. C. Davis¹ and L. E. Erickson²

Department of Biochemistry¹
Department of Chemical Engineering²

Abstract

Tetrachloroethene (PCE) is a major cause of organic groundwater pollution, primarily because of its disposal after use as an industrial cleaner. Microcosms were prepared with contaminated ground water taken from below a former dry cleaner facility in Manhattan KS. Amendments such as soy oil methyl esters, yeast extract and cheese whey were used as nutrients to stimulate microbial dechlorination of PCE, while the anaerobic bacterial culture KB-1 was used in bioaugmentation studies. Results show that microcosms treated with KB-1 culture and amended with nutrients were capable of reducing the PCE concentrations ≥90%. Yeast extract and soy oil methyl esters were capable of creating reducing conditions to dechlorinate PCE so that cis-DCE and methane were produced. In cheese whey experiments, the KB-1 culture was inhibited with concentrations above 0.05% of whey. This inhibition was due to formation of acid that lowered the pH and inhibited the activity of the bacterial culture. On the other hand, low concentration of cheese whey (0.025%) stimulated contaminant reduction and methane generation. These results suggest that bioaugmentation and biostimulation are potent alternatives for PCE degradation and aquifer remediation.

Introduction

Tetrachloroethene also known as perchloroethylene (PCE) and its degradation product trichloroethene (TCE) are widely used in dry-cleaning and other industries. Unfortunately, nonproper storage and handling practices have lead to their leaking into the environment. They are among the most common contaminants of soil and groundwater (Magnuson et al, 1998, Lee et al, 1998, Furukawa K., 2006).

Conventional remediation approaches for soil and ground water remediation such as chemical oxidation, soil vapor extraction or pump and treat were used for treating sites contaminated with chlorinated solvents, but physical properties of PCE such as low solubility and high density makes it very difficult to treat. Therefore, the use of in situ biological treatment (bioremediation) appears to be a viable and cost effective alternative (Muller et al, 2004). A number of bacterial cultures have been isolated with ability to reductively dehalogenate chlorinated compounds in a process called dehalorespiration (Suyama et al, 2001). Current evidence suggests that PCE is recalcitrant to aerobic degradation, and can only be removed by anaerobic reductive dechlorination processes (Kao et al, 2003). An anaerobic consortium of bacterial culture KB-1 was used in this study. KB-1 contains Dehalococcoides ethenogenes which is capable of complete dechlorination of PCE to safe end products methane and ethene (Duhamel et al, 2004). A number of organic compounds such as acetate, methanol (Major et al, 2002),
glucose, phenol, molasses, yeast extract, and vegetable oils (OPPTD Document, 1217) were used as electron donors or as carbon source to stimulate growth of degrading organisms. This study addresses the use of cheese whey as a nutrient and electron source for microbial biostimulation, for it contain proteins, sugars (mainly lactose) and vitamin B12 (Tomasula et al. 1998 & Bullerman L.B., and Berry E.C., 1966). Therefore, the objective of this study is to evaluate biostimulation and bioaugmentation technologies in PCE remediation in soil and groundwater.

Site Characterization

A former dry cleaner site in Manhattan, KS has contaminated the soil and ground water with PCE during 30 years of operation. Currently the site lies under a commercial area with paved parking lot down gradient of the contaminated source as shown in Figure 1. Studies done by Kansas Department of Health and Environment (KDHE) and their contractors revealed a contaminated ground water plume. Chlorinated solvents were detected above their maximum contamination levels (MCL) threatening the public water wells located 1.5 miles east of the contaminated zone. Several monitoring wells were installed along the plume to monitor concentrations of PCE and its degradation products. In monitoring wells close to source, PCE was the main contaminant found at a concentration up to 25 mg/L. Down-gradient, PCE was found with trichloroethylene (TCE) and cis-dichloroethylene (DCE) at a concentration of 7.5 mg/L. Vinyl chloride was rarely detected (Terracon, 2004). Water samples used in microcosms were taken from monitoring well-5, located near the source area and monitoring well-8, about 80 ft down-gradient (see Figure 1).
Microcosms were prepared in 16.5 ml vials sealed with Teflon mininert caps. Water samples were collected from monitoring wells located at the contaminated site. Different concentrations of cheese whey were used in this study, ranging from 0.01%-0.5%. Microcosms with yeast extract and soy oil methyl esters were prepared and compared to cheese whey. In a separate study cheese whey was filter sterilized through 0.22 μm Millipore (nitrocellulose) filter paper and used to study effect of sterile vs natural microflora of cheese whey on KB-1 culture. In all microcosms the final volume of liquid phase was 12 mL and resazurin was added to all bottles to monitor redox conditions. Nitrogen gas was used to flush the vials for 30 seconds, which were then closed to prepare for anaerobic conditions. Control samples contained only contaminated water (no nutrients and no KB-1). Vials were kept at room temperature and 10 μl of KB-1 was added after the Resazurin color changed from blue to pink to colorless as an indication of anaerobic conditions.

Analytical Method

Chlorinated ethenes and methane were analyzed using the HP 5890 series II gas chromatograph (Hewlett-Packard, Wilmington, DE) equipped with HP-1 column (Dimethyl Polysiloxane matrix, 30 m x 0.53 mm, Agilent Technologies) and flame ionization detector. Hydrogen was the carrier gas. Injector temperature was set at 200°C and detector temperature at 300°C. The 100 μL samples were manually injected. An Isothermal program was created and temperature was set in the column at 100°C and run time was 5 minutes. To resolve methane from ethene, a thermal conductivity GC was used (Carle 8501) with a 1.83m x 3.2 mm porapak S column at 80 °C.
Results and Discussion

Nutrient biostimulation tests were carried out with soy oil methyl esters, yeast extract, or cheese whey. Results show that natural microorganisms in the aquifer were able to carry out PCE degradation up to DCE level. DCE accumulation was observed for several months (Fig. 2). Bioaugmented microcosms were prepared with bacterial culture KB-1 containing *Dehalococcoides ethenogenes*. Bacterial culture amended with nutrients was able to convert more than 90% of chlorinated ethenes into methane as a final end product (Fig. 3). When the yeast extract and soy oil methyl ester treatments were compared with low concentrations of cheese whey (< 0.1%) there was no difference in the rate of PCE degradation; and a lag phase of 10-20 days was observed. Higher concentrations of cheese whey, 0.1% and 0.25%, did not differ from control microcosms. Bacterial Inhibition was not due to competition between cheese whey microbial culture and KB-1 (Fig. 4). Evidence is shown in Figure 5 where filter sterilized cheese whey was used to amend KB-1 culture and inhibition was still recorded with high cheese whey concentrations (0.25% and 0.5%).

![Figure 2](image-url)

**Figure 2.** Biostimulation experiment with water from top of monitoring well 5, amended with 0.05% cheese whey.

![Figure 3](image-url)

**Figure 3.** Biostimulation and bioaugmentation experiment with 0.05% cheese whey and 10 μl of KB-1 culture. Complete reduction in chlorinated ethenes, PCE was first reduced to DCE followed by further dechlorination of DCE to methane. KB-1 was added in day 3 after establishing reducing conditions.
Figure 4. Bioaugmentation experiments with KB-1 culture. Comparison between nutrient amendments of cheese whey and a combination of soy oil methyl ester and yeast extract. Microcosms prepared with water taken from monitoring well 5.

Figure 5. Cheese whey inhibition experiment. Filter sterilized cheese whey used to prepare microcosms from monitoring well 5. Only low cheese whey concentrations (0.05% and 0.03%) show active reduction in chlorinated ethenes.
Conclusion

Biodegradation of tetrachloroethene in contaminated ground water was possible under suitable nutrient and reducing conditions. KB-1 bacterial culture can reduce chlorinated ethenes to safe end products methane or ethene under anaerobic conditions. DCE accumulates in the systems that contain the endogenous microflora from the contaminated site. Cheese whey is an effective source of nutrients and electron donor if used at low concentrations (<0.1%), and showed similar results as soy oil methyl ester and yeast extract combination treatments. Inhibition at higher concentrations of cheese whey was later found to be from acid accumulation in the vials and not related to microbial competition between cheese whey and KB-1 culture.

Acknowledgment

Thanks to Kansas Department of Health and Environment for funding this project, Dr. Stacy Hutchinson for use of lab space and GC, Sirem Laboratories for providing free samples of KB-1 for this study and Dr. Fadi Al-Aramouni for cheese whey.

References


Development of Genetically Engineered Biosealants
Terran J. Elliott and Sookie S. Bang
Department of Chemical and Biological Engineering
South Dakota School of Mines and Technology
Rapid City, SD, USA 57702

Abstract
The purpose of this research was to develop genetically engineered microorganisms capable of inducing calcite precipitation and producing an extracellular polymer substance for enhanced crack remediation. A new plasmid, pUBU1, was constructed with the vector pUCP18 and the gene sequences encoding urease from plasmid pBU11. Plasmid pBU11 contains the urease genes from Bacillus pasteurii, which are essential for subsequent calcite precipitation. Plasmid pUCP18 is a shuttle vector for Escherichia coli and Pseudomonas. The EPS producing microorganism, Pseudomonas aeruginosa, was transformed with the plasmid, through electroporation, resulting in recombinant strains P. aeruginosa 8821 (pUBU1) and P. aeruginosa PA01 (pUBU1). Results indicate that the recombinant microorganisms are able to express the urease genes, induce calcite precipitation, and form biofilms comparable to the wild-types.

Introduction
Microbiologically induced calcium carbonate, CaCO₃, precipitation (MICCP) consists of a series of complex biochemical reactions. During the process the microorganism plays a key role by producing the enzyme urease (urea amidohydrolase, E.C. 3.5.1.5), which hydrolyzes urea into ammonia and carbon dioxide (Rxn I) to increase the surrounding pH. The CO₂ is ionized to carbonate (Rxns II-III), and reacts with Ca²⁺ ions to precipitate as CaCO₃ (Rxn IV) in the calcite form (Stocks-Fischer et al. 1999). The solubility of CaCO₃ is a function of pH: solubility decreases as the pH increases.

\[
\begin{align*}
\text{NH}_2 - \text{CO} - \text{NH}_2 + \text{H}_2\text{O} \xrightarrow{\text{urease}} & 2\text{NH}_3 + \text{CO}_2 \quad (I) \\
\text{NH}_3 + \text{CO}_2 + \text{H}_2\text{O} & \rightarrow \text{NH}_4^+ + \text{HCO}_3^- \quad (II) \\
\text{HCO}_3^- + \text{H}_2\text{O} & \rightarrow \text{H}_3\text{O}^+ + \text{CO}_3^{2-} \quad (III) \\
\text{Ca}^{2+} + \text{CO}_3^{2-} & \rightarrow \text{CaCO}_3 \text{ (ppt)} \quad (IV)
\end{align*}
\]

It has been reported that several organisms (Fujita et al. 2000, Rodriguez-Navarro 2003) can produce this type of MICCP, but for this research project the focus was directed towards Bacillus pasteurii. Microscopic examination of MICCP identified that the bacterium served as a nucleation site during the process (Stocks-Fisher et al. 1999). It was also proposed that an increase of pH across the cell membrane due to the ammonia production induces a localized calcite precipitation on the cell surface. Further examination of MICCP by B. pasteurii showed that the process effectively remediated concrete cracks and increased the compressive strength by 61% in cement mortar cubes (Ramachandran et al. 2001). Because urea is relatively inexpensive and enzymatically hydrolyzed into innocuous compounds, CO₂ and NH₃, it is considered an ideal substrate to be used in MICCP for subsequent crack remediation. MICCP occurs within the crack and increases compressive strength, but is unlikely to induce any chemical bonding between CaCO₃ and the cement matrix (Bang et al. 2000). To discover a more
effective sealant and induce this type of chemical bonding, the use of biopolymer production in addition to MICCP was proposed (Bang 2004).

The specific role of microbial urease in MICCP was identified using *Escherichia coli* HB101 (pBU11) whose plasmid codes for the *B. pasteurii* urease operon (Kim and Spizizen 1985) and also contains the pBR322 vector (Bolivar 1977). The urease operon contains three structural genes (ureABC) (Benini *et al*. 1999) and four accessory genes required for nickel incorporation into the apourease, which is needed for the expression of urease as a holoenzyme (McGee *et al*. 1999). The pBU11 plasmid has a length of 4,361 bp and is only able to replicate in *E. coli*. Therefore, efforts were made to seek vectors with a broader host range, allowing its replication in both *E. coli* and *P. aeruginosa*.

Vector pUCP18 (Schweizer 1991) is a pMB1(ColE1)-based plasmid and can replicate in *Pseudomonas* due to the presence of a stabilizing fragment of a pRO1600-derived portion of pRO1614 (Olsen *et al*. 1982). The 1.9-kbp *PstI* fragment of pRO1614 permits stable replication of this vector in *Pseudomonas*. The pUCP18 vector has a total length of 4,557 bp, including a *lacZ* gene and an ampicillin resistance gene.

Some bacteria are known to form biofilms and extracellular polymeric substances (EPS) for enhanced survival (Banin *et al*. 2006). *P. aeruginosa* is one such bacterium and is known to become extremely mucoid, due to the synthesis and secretion of the EPS alginate that is composed of guluronic and mannuronic acid residues (May and Chakrabarty 1994). High levels of alginate production are characteristic of the *P. aeruginosa* 8821 strain that is a clinical isolate, but not of the PA01 strain that is an environmental isolate. In the *P. aeruginosa* PA01 biofilm matrix, an EPS composed of glucose, rhamnose, and mannose appears to serve as the backbone for DNA and proteins (Matsukawa and Greenberg 2004). Both of these *Pseudomonas* species produce biofilms and EPS with adhesive properties that could augment the plugging characteristics of MICCP. The EPS could serve as an adhesive sealant and increase the strength of structures they are applied to. Therefore, we proposed to induce urease production in *P. aeruginosa* and create a new recombinant microorganism for microbiologically enhanced crack remediation (MECR).

The concept that the recombinant will enhance remediation is suggested through several studies indicating that Ca$^{2+}$ ions are chelated by biofilms and EPS. Banin *et al*. (2006) found that EDTA caused dispersal of *P. aeruginosa* and that calcium was involved in biofilm maintenance. Sarkisova *et al*. (2005) demonstrated that increasing the calcium concentration by adding 10 mM CaCl$_2$ resulted in a 20-fold thicker biofilm of mucoid *P. aeruginosa* than those without calcium added. Nuclear magnetic resonance studies also suggested that the alginate chain provided binding sites for Ca$^{2+}$ ions through chelation (Moe *et al*. 1995). Therefore biofilm production is enhanced by the presence of calcium ions and the EPS provides a specific attachment sight for these ions. It is hypothesized in the current study that the presence of Ca$^{2+}$ in the medium will support biofilm formation and that the binding sites for Ca$^{2+}$ ions may serve as nucleation sites for CaCO$_3$ crystal formation in the EPS matrices of recombinants. This hypothesis is supported by suggestions from others indicating the role of EPS in trapping ions (Ercole *et al*. 2007).

Figure 1 depicts the overall scheme of this research including construction of recombinant *Pseudomonas* strains that are capable of inducing CaCO$_3$ precipitation and producing EPS. As indicated the isolation of urease genes from *B. pasteurii* was accomplished by Kim and Spizizen (1985). Through the current work a new plasmid, pUBU1, was constructed for the preparation of the *Pseudomonas* recombinants. This new plasmid harbors the urease genes of *B. pasteurii*.
Methods and Results
Bacterial strains, plasmids, and media.

The bacterial strains and the plasmids used in this study are listed in Table 1. *E. coli* and *P. aeruginosa* were grown in Luria-Bertani (LB) broth at 37°C. *B. pasteurii* ATCC 11859 was grown in medium ATCC 1832 at 30°C. Plasmids were maintained with antibiotics in the following concentrations: 100 μg/ml ampicillin in *E. coli* species, 300 μg/ml carbenicillin in *P. aeruginosa* species. To ensure urease activity, NiCl₂ (50 μM) was supplemented for urease-encoding plasmids.

Table 1. Bacterial strains and plasmids used in this study.

<table>
<thead>
<tr>
<th>Plasmids or Strains</th>
<th>Relevant Characteristics</th>
<th>Source or Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasmids</td>
<td></td>
<td></td>
</tr>
<tr>
<td>pBU11</td>
<td>derived from pGU66 by inserting a 10.7 kbp of HindIII fragment into the (pBR322)</td>
<td>Kim and Spizizen (1985)</td>
</tr>
<tr>
<td>pBR322</td>
<td>Ap⁺, Tef'</td>
<td>Bolivar et al. (1977)</td>
</tr>
<tr>
<td>pUCP18</td>
<td><em>E. coli</em>-Pseudomonas shuttle vector derived from pUC18/19; ColE1, pRO1600 replicon, lacZ, bla</td>
<td>Schweizer (1991)</td>
</tr>
<tr>
<td>pUBU1</td>
<td>Derived from pBU11 by inserting a 12kbp HindIII fragment into the pUCP18 vector</td>
<td>This work</td>
</tr>
<tr>
<td>Strains</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>E. coli</em> HB101</td>
<td>F⁻, thi⁻, hsdS20 (rF⁻, mR⁻), supE44, recA13, ara-14, leuB6, rolA2, lacY1, galK2, rpsL20 (str'), xyl-5, mtl-1</td>
<td>Lacks and Greenberg (1977)</td>
</tr>
<tr>
<td><em>E. coli</em> JM109</td>
<td>endA1, recA1, gyrA96, thi, hsdR17 (r5⁻, m4+), relA1, supE44, Δ(lac-proAB), [F' traD36, proAB, lacF*Z Δ M15]</td>
<td>Yanisch-Perron et al. (1985)</td>
</tr>
<tr>
<td><em>Bacillus pasteurii</em></td>
<td>Wildtype, urease positive</td>
<td>ATCC 11859</td>
</tr>
<tr>
<td><em>P. aeruginosa</em> 8821</td>
<td>his-1 alg+, mucoid CF isolate</td>
<td>Darzins and Chakrabarty (1984)</td>
</tr>
<tr>
<td><em>P. aeruginosa</em> PAO1</td>
<td>Prototroph, non-mucoid</td>
<td>ATCC 15692</td>
</tr>
</tbody>
</table>

Construction of pUBU1 plasmid.

Figure 2 depicts this plasmid construction including the complete digestion, which produces undesirable fragments that do not contain the entire urease operon, and partial digestion possibilities. Three possibilities, including the desirable 11.92-kbp fragment encoding the entire urease sequence, are shown. The pUBU1 plasmid was constructed through ligation of a partially digested *HindIII* fragment (11.92-kbp) of pBU11 with the pUCP18 vector. Once the desirable
fragment was isolated and purified, it was ligated with the pUCP18 vector and inserted into competent *E. coli* JM109 cells for continuous replication.

After construction of the new plasmid, restriction enzyme digestions were performed on both the pBU11 and the new pUBU1 plasmid. In Figure 3a the pBU11 and pUBU1 plasmids were digested with *BamHI* and the patterns shown through gel electrophoresis. Plasmid pBU11 was digested into 3 fragments, with sizes of 8.15-kbp, 4.63-kbp, and 3.50-kbp. The pUBU1 plasmid produced the same 3.50-kbp band found in pBU11, but in pUBU1 a 12.3-kbp band and a small band, 0.65-kbp, were observed.

![Diagram of plasmid construction](image)

**Figure 2.** New plasmid construction scheme from pUBU1 using partial digestion.

**Figure 3.** Comparison of gel electrophoresis patterns of plasmids, pBU11 (Lane 2) and pUBU1 (Lane 3), treated with *BamHI* (a) and *BamHI* and *StuI* (b). Lane 1 contains a 1-kbp molecular weight marker, where the thickest band corresponds to 5-kbp. Values at the arrows indicate fragment molecular weights as kbp.
Plasmids pUBU1 and pBU11 were further analyzed by the double digestion with the restriction enzymes *BamHI* and *StuI* (Figure 3b). Vector pUCP18 of pUBU1 has a *StuI* restriction site that is not present in the pBR322 vector of pBU11. From the double digestion with *BamHI* and *StuI*, pUBU1 would theoretically display an additional fragment. In Figure 3b, the pUBU1 plasmid produces fragments of 9.23-kbp, 3.50-kbp, 3.10-kbp, and 0.65-kbp (Lane 3), however pBU11 (Lane 2) shows the same pattern observed upon single digestion with *BamHI* (Figure 3a) as predicted.

Figures 4 and 5 depict the restriction maps of pBU11 and pUBU1. These maps were developed by restriction enzyme mapping of the two plasmids using the specific enzymes indicated on the map. *B. pasteurii* urease and the vector sequences were found in the NCBI GenBank and uploaded into the mapping software, Invitrogen Vector NTI (www.invitrogen.com), as well as the fragment sizes observed from restriction digestions. Through the mapping process the sizes of the plasmids were determined to be 16.28-kbp for pBU11 (Figure 4) and 16.48-kbp for pUBU1 (Figure 5).

**Figure 4.** Plasmid pBU11 restriction map. **Figure 5.** Plasmid pUBU1 restriction map

**Development of Recombinants**

*Pseudomonas* recombinant microorganisms were prepared by introducing the pUBU1 plasmid into strains of *P. aeruginosa* 8821 and PAO1 by electroporation. Figure 6 depicts a schematic for the development of recombinant *Pseudomonas* microorganisms. First the plasmid was extracted from the *E. coli* JM109 host and then transformed into the EPS producing *Pseudomonas* strains through electroporation to produce the new recombinants.

**Figure 6.** Transformation scheme of pUBU1 into *Pseudomonas* strains to construct recombinants.
These recombinants were indicated as *P. aeruginosa* 8821 (pUBU1) and *P. aeruginosa* PA01 (pUBU1). Initial tests involved observing mucoidy and pH increases after inoculation in urea phenol broth. To confirm the new recombinants were properly developed, the plasmid was re-extracted from the individual *Pseudomonas* species. Figure 7 shows the recovery of the pUBU1 plasmid from the recombinant microorganisms. Zwitterion detergent solution was required to remove the EPS layer of *Pseudomonas* cultures and to improve gel electrophoresis (Domenico *et al.* 1992). However, some streaking was still apparent (Figure 7).

Lane 1 contains the 1 kb marker. The *E. coli* JM109 (pUBU1) plasmid is shown in Lane 2, where two bands are visible: the upper band is linear or uncoiled plasmid DNA due to nicking of supercoiled DNA in the preparation process, the lower band is supercoiled plasmid DNA. In Lane 3, a cryptic plasmid (MW~20-kbp) is found to be inherent in the *P. aeruginosa* 8821 strain. The recombinant, *P. aeruginosa* 8821 (pUBU1) in Lane 4 also has the cryptic plasmid as well as the pUBU1 plasmid. Currently no other known research has found this cryptic plasmid or transformed this specific strain with a self-replicating plasmid. It is proposed that this cryptic plasmid of approximately 20-kbp may be similar to a plasmid found in the *P. aeruginosa* 8822 strain, which is a spontaneous non-mucoid derivative of strain 8821 (Darzins and Chakrabarty 1984). In communication with Dr. Chakrabarty (2006), the plasmid found in the *P. aeruginosa* 8822 strain was suggested to be a chromosomal fragment that gets circularized and excised at a low frequency to look like a plasmid. Currently, it is not known whether the plasmid found in *P. aeruginosa* 8821 is similar to the one found by Chakrabarty in *P. aeruginosa* 8822.

![Lane 1 Lane 2 Lane 3 Lane 4 Lane 5 Lane 6](pUBU1 8821 8821 (pUBU1) PAO1 (pUBU1))

**Figure 7.** Gel electrophoresis of plasmids isolated from *Pseudomonas* strains.

Due to the potential pathogenicity of *P. aeruginosa* 8821, *P. aeruginosa* PAO1 was used to develop another recombinant for MECR. The PAO1 recombinant strain was found to be visibly capable of producing biofilms. However, the cryptic plasmid found in *P. aeruginosa* 8821 is absent in *P. aeruginosa* PAO1 (Lane 5, Figure 7) and *P. aeruginosa* PAO1 (pUBU1) (Lane 6, Figure 7). Overall, Figure 7 shows that each *Pseudomonas* recombinant has been successfully transformed and capable of continuously replicating the pUBU1 plasmid.
Identification of Recombinants

Calcium Carbonate Precipitation: After developing the recombinant microorganisms it was essential to quantify their ability to precipitate calcite, as established by Stocks-Fischer et al. (1999). To avoid difficulty with color development, it was necessary to add the inhibitor II reagent prior to titration of the insoluble Ca\(^{2+}\) (American Water Works Association 1998). Results showed a clearly detectable color change throughout the experiment. It is possible that a color inhibition occurred due to the ability of the EPS to chelate other metal ion besides Ca\(^{2+}\), which could interfere with the titration. Pseudomonas species are known to produce polyphosphate under various conditions (Zago et al. 1999) that may cause interference with the titration. The inhibitor II reagent is able to complex with metals and polyphosphate prior to titration to eliminate their effects on the titration.

The results of CaCO\(_3\) precipitation of Pseudomonas recombinant species and the wild types are summarized in Table 2. The initial rates of precipitation were not as fast as the B. pasteurii (data not shown), however almost all of the available calcium ions (25.25 mM) were converted to the insoluble form after 24 hours by both recombinants (Table 2).

<table>
<thead>
<tr>
<th>Strains</th>
<th>Insoluble Ca(^{2+}) (mM)</th>
<th>Urease activity (units/mg protein)</th>
<th>Alginate (mg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>B. pasteurii ATCC 11859</td>
<td>25.25</td>
<td>127.44</td>
<td>ND</td>
</tr>
<tr>
<td>P. aeruginosa 8821</td>
<td>---</td>
<td>---</td>
<td>119.9</td>
</tr>
<tr>
<td>P. aeruginosa PA01</td>
<td>---</td>
<td>---</td>
<td>3.03</td>
</tr>
<tr>
<td>P. aeruginosa 8821 (pUBU1)</td>
<td>25.12</td>
<td>48.45</td>
<td>120.12</td>
</tr>
<tr>
<td>P. aeruginosa PA01 (pUBU1)</td>
<td>25.08</td>
<td>50.51</td>
<td>ND</td>
</tr>
</tbody>
</table>

Enzyme Activity: Numerous trials were performed to determine the specific enzyme activity. Initial results were inconsistent with relatively high standard deviations and very low values for the recombinant species (data not shown). Based on the fact that the plasmids have a high copy number from the pUCP18 vector (Schweizer 1991) and the results of the calcite precipitation, the enzyme activities of the recombinants were expected to be comparable to B. pasteurii. P. aeruginosa 8821 (pUBU1) and P. aeruginosa PA01 (pUBU1) had specific enzyme activities that were 38% and 40% of the enzyme activity of B. pasteurii activity, respectively. These results seem to indicate that the urease activity of B. pasteurii exceeds the required concentration for calcite precipitation, as the recombinants did not produce as much urease as B. pasteurii but were still capable of inducing calcite precipitation at comparable levels.
Alginate Determination: As shown in Table 2, the recombinant microorganism *P. aeruginosa* 8821 (pUBU1) produces a comparable level of alginate activity to the wild-type, *P. aeruginosa* 8821. An insignificant level of alginate was detected in *P. aeruginosa* PA01. This finding is in accordance with the results of Wozniak *et al.* (2003), where a low level of alginate activity was found with the *P. aeruginosa* PA01 strain. However, the strain was capable of forming a biofilm and producing an EPS composed of no alginate.

Scanning Electron Microscopy (SEM): Electron micrographs of the recombinant strains (8821 and PA01) of *Pseudomonas* were compared with those of *B. pasteurii* and wild-types of *P. aeruginosa*. Figure 8 depicts the SEM images taken for the wild-type and recombinant microorganisms grown in urea-CaCl$_2$ medium. Figure 8a shows the *P. aeruginosa* wild type, and depicts the biofilm and alginate-dominated EPS growth on the glass. In Figure 8b *B. pasteurii* is found to produce characteristic rhombohedral crystals, which aggregate into spherical and semi-spherical structures. In Figure 8c, calcite crystals formed by *P. aeruginosa* 8821 (pUBU1) aggregate into spherical and flower-like structures, which appear to be embedded in the EPS layers. *P. aeruginosa* PA01 (pUBU1) is depicted in Figure 8d, where the EPS appears to fully encase all the calcite crystals.

![Figure 8. SEM images of calcite crystals and EPS produced by the wild types and recombinants. a. *P. aeruginosa* 8821, b. *B. pasteurii* ATCC 11859, c. *P. aeruginosa* 8821 (pUBU1), d. *P. aeruginosa* PA01 (pUBU1).](image_url)

Final Conclusions

The main goal of this research was to develop recombinant microorganisms to augment MICCP. Two different recombinant *Pseudomonas* species capable of inducing CaCO$_3$
precipitation and producing EPS were developed. These recombinants were prepared by electroporation of the newly constructed pUBU1 plasmid into the EPS producing strains. Interestingly, the *P. aeruginosa* 8821 strain was found to produce a cryptic plasmid, which does not appear to affect the activity of the pUBU1 plasmid in terms of replication or ability to produce urease. The recombinants were found to express urease at levels ~40% that of *B. pasteurii*. Although the specific enzyme activity was not as high as the wild-type, calcite precipitation was induced by the recombinants at comparable rates to *B. pasteurii*. This indicates the amount of urease produced by the wild-type exceeds the amount needed for MICCP. The ability for the recombinants to produce EPS might also have contributed to the high rates of MICCP, where recombinant *P. aeruginosa* 8821 (pUBU1) produced comparable alginate levels to *P. aeruginosa* 8821. SEM analysis also showed that the CaCO$_3$ crystals appeared to be encased within the EPS matrix of the recombinant *Pseudomonas* strains.

Preliminary results indicated that these recombinants have potential to provide a biosealant for MECR. Future work will involve examination of the ability for these recombinant microorganisms to work as biosealants in concrete cracks. We plan to apply the recombinant microorganisms into concrete fissures and measure the compressive strengths. It is hypothesized that the newly constructed recombinants will enhance the strength through bonding of the biofilm to the surface and the calcite will provide compressive strength as seen previously with *B. pasteurii*.

**Acknowledgements**

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**References**

Use of Statistical Design for the Optimization of Protein Expression in Baculovirus Expression Vector System

Alexander Brix1, Bernd Eichenmueller2, Peter Czermak1,3

1University of Applied Science Giessen-Friedberg, Giessen – Germany
2Boehringer Ingelheim Vetmedica, Inc., St. Joseph, MO - USA
3Kansas State University, Dept. of Chemical Engineering, Manhattan, KS - USA

Abstract

The objective of this work is to improve the protein production processes using statistical experimental design to obtain a consistent and acceptable protein yield. Insect cells are used as biological host cells for the baculovirus expression vector system (BEVS). All experiments are performed in Applikon® BioClave 20 L bioreactors. The effects of process parameters such as agitation rate, aeration rate and dissolved oxygen level on the protein yield is investigated. Using a statistical design of experiments is more efficient than the common one-factor-at-a-time or brute-force approaches. It allows to minimize the number of experiments and to estimate an interaction between different parameters and the protein yield at the same time.

Materials & Methods

Experimental Design
In this work the effect of three process parameters with three levels each (low: -1, center: 0, high: +1) is investigated. Therefore the experimental design chosen in this work is a two level full factorial approach with a total of 12 experiments. Process parameters are set to low (-1) and high (+1) level and run in every possible combination. Additionally four centerpoint experiments with process parameters set to the average value of low and high level (center: 0) are performed. This design is more efficient than the commonly used one-factor-at-a-time or brute-force approaches. It allows determination of interactions between the process parameters at a comparatively low number of experiments.

Cell Line and Baculovirus Expression Vector System
A suspension cell line derived from Spodoptera frugiperda Sf9 insect cells is used as biological host for the baculovirus expression vector system (BEVS) and the production of the target-protein.

Bioreactor
All experiments are performed in an Applikon® BioClave 20 L autoclavable stainless steel bioreactor. The bioreactor is controlled by a combination of Applikon® ADI 1030 BioController and ADI 1035 BioConsole. The vessel is equipped with two marine impellers and a sparge tube underneath the lower impeller.
ELISA

Target-protein concentration is measured by Sandwich Enzyme Linked Immunosorbent Assay. The particular result is indicated as Relative Potency (RP) to a reference with known target-protein concentration.

Realization and Analysis

Insect cells are maintained in spinner vessels. The bioreactor is inoculated at a specific planting cell density and the cells are grown using identical process parameter setpoints for each bioreactor run. After the cells have reached a particular density they are infected with baculovirus seed at a constant multiplicity of infection (MOI). During the following protein expression phase the three chosen process parameters agitation rate, aeration rate and dissolved oxygen level are set to their particular new setpoints according to the experimental design.

Samples are taken during cell growth phase and target-protein production phase for cell count, cell viability and metabolite concentrations. Samples taken after infection are additionally tested for target-protein concentration utilizing ELISA. Data analysis is performed with software MODDE (Umetrics).
Modeling Pore Size Distribution of Ultrafiltration membranes

A. Mukherjee¹, S.R. Wickramasinghe¹

¹Department of Chemical and Biological Engineering, Colorado State University, Fort Collins, CO 80523, United States of America, wickram@engr.colostate.edu

Abstract

Ultrafiltration is a low pressure (5-150 psig) membrane separation process used in various industries to separate solutes from a solution. Industrial applications include pharmaceutical production, food processing, nutraceutical production and virus clearance among others. The nominal molecular weight limit (NWML) of ultrafiltration membranes range from 1-1000 kDa and pore sizes are typically in the range 10 to 1000 Å.

It is desirable to know how membrane morphology affects the performance of the membrane. It is a known fact that membrane pores do not have a uniform diameter. The pore size distribution (PSD) is thus the key parameter of interest for membrane characterization. The ultimate challenge will however lie in tailoring the PSD to match the requirements of a given separation.

In this paper we have used Field Emission Scanning Electron Microscopy (FESEM) to visualize polyether sulfone and regenerated cellulose ultrafiltration membranes having molecular weight cutoffs 100, 300, 300, 1000 kDa respectively.

A mathematical model was used to determine the PSD and rejection. A lognormal distribution was used to fit the PSD data and model the Dextran rejection profile.

1. INTRODUCTION

Ultrafiltration is a low pressure (5-150 psig) membrane separation process used in various industries to separate solutes from a solution. Industrial applications include pharmaceutical production, food processing, nutraceutical production and virus clearance among others.

Ultrafiltration employs porous membranes to separate solutes. Separation is based on molecular size. If solute diameter is less than pore diameter, the solute passes through the membrane. Solutes with diameter nearly equivalent to the pore diameter permeate partially whereas the passage of larger solutes is blocked. The sieving coefficient of an ultrafiltration membrane for a given solute is defined as the ratio of the solute concentration in the permeate to the solute concentration in the feed. The rejection coefficient ranges between zero and one and is equal to one minus the sieving coefficient. A value of zero implies complete permeability of the feed solution whereas a rejection coefficient equal to one implies total rejection. The molecular weight of the solute corresponding to a rejection coefficient of 0.9 has been universally accepted to designate the net molecular weight limit (NMWL) of an ultrafiltration membrane. The rejection coefficient depends on not only the pore size distribution and the solute radius but also on membrane solute interactions and mass transfer characteristics of the membrane [1].
The (NMWL) of ultrafiltration membranes range from 1-1000 kDa and pore sizes are typically in the range 10 to 1000 Å. A large spectrum of polymers has been used to manufacture ultrafiltration membranes such as cellulose acetate (CA), polysulfone (PS), Polyether Sulfone (PES), Polyvinylchloride, etc.

Ultrafiltration membranes can be symmetric or asymmetric. The symmetric membranes are constructed out of a single material. In asymmetric membranes the functional layer is grafted on to a thicker support structure. The functional layer contains the ultrafiltration pores while the structural layer provides mechanical strength. Integrity testing and backpressure testing is carried out to determine the mechanical strength. The pores on the support structure are much larger than the ones present on the functional layer and are usually greater by an order of magnitude [2]. Thus the support layer has no effect on membrane performance which is governed only by the functional layer.

The NMWL shows a lot to lot variability and also membranes from different manufacturers with the same claimed NMWL can exhibit substantially different rejection for the same solute. Membranes do not have uniform pores. Rather there exists a pore size distribution. A pore size distribution is a measure of the relative frequency of pores with different diameters present per unit cross sectional area of the membrane. The NMWL is governed by the larger pores on the membrane surface. The greatest challenge lies in developing a method for the manufacture of membranes with a tailored uniform pore size distribution to suit a particular separation. Thus as a first step determination of pore size distribution of different membranes is of great interest. There are a number of reviews on membrane pore characterization in the literature [3-5]. Nakao has reviewed the existing methods for determining pore size and pore size distribution of filtration membranes [6]. Youm et.al have studied the effect of operating conditions on pore properties [7].

The methods for determining pore size distribution can be broadly classified as direct and indirect methods. Direct methods involve microscopic visualization of membrane surfaces followed by image processing and analysis. The most widely used direct methods are Scanning Electron Microscopy (SEM), Atomic Force Microscopy (AFM) [8-11]. Transmission Electron Microscopy (TEM) [12-14] and Small-Angle Neutron Scattering [15] have also been used. SEM is favored over TEM due to the higher resolution achieved and the comparative ease of sample preparation. Even with SEM there is the problem of surface damage as the electron beam has high energy. However Field Emission Scanning Electron Microscopy (FESEM) is now the method of choice as a high resolution can be achieved at accelerating voltages in the range of 1.5-4kV [16-18]. Indirect methods measure a secondary effect of the membrane which is then correlated to the pore size distribution. Some of the methods are Gas Transport, Thermoporometry, Liquid displacement, Permporometry, etc [19-22]. Table 1 compares some of these methods.
Table 1: Indirect methods of determining Pore Size Distribution

<table>
<thead>
<tr>
<th>METHOD</th>
<th>PRINCIPLE</th>
<th>PARAMETER MEASURED</th>
<th>ADVANTAGES</th>
<th>DISADVANTAGES</th>
</tr>
</thead>
<tbody>
<tr>
<td>Permporometry</td>
<td>Capillary condensation of a liquid inside a porous medium</td>
<td>Pore Size Distribution</td>
<td>1. Only active pores are characterized&lt;br&gt;2. No Fouling</td>
<td>1. Extremely simplified model&lt;br&gt;2. Requirement of a dried membrane&lt;br&gt;3. Measured curves dependent on chemical nature of condensing material[23]</td>
</tr>
<tr>
<td>Liquid-Liquid Displacement</td>
<td>Two immiscible liquids used. One is used to fill the pores and the other to displace the first. Pore Size Distribution determined from flow versus versus pressure plots</td>
<td>Pore Size Distribution</td>
<td>1. Functional pores characterized&lt;br&gt;2. Easy automation</td>
<td>1. Model Dependent&lt;br&gt;2. Membrane swelling</td>
</tr>
</tbody>
</table>

Recently ultrasonic frequency domain reflectometry, Raman spectroscopy, electron spin resonance and light transmission combined with the bubble pressure method have also been used
to determine the pore size distribution [25-28]. In general both direct and indirect methods are used simultaneously to determine the pore size distribution and other membrane characteristics such as void fraction, surface porosity, etc.

The experimental result from a direct pore size measurement of a membrane surface is a discrete pore size distribution of the observed surface pores. This pore size distribution is assumed to remain similar throughout the bulk of the membrane. The experimental data from indirect pore size measurement experiments need to be fitted to a nominal pore size distribution model. Different distribution functions have been used to relate solute size and solute rejection in order to characterize the pore size distribution. The choice of the pore size distribution model has a significant impact on the quality of the fit results [29-31].

Green et al. [32] had proposed the Gaussian distribution function to relate sieving coefficients to electron spin microscopy. However the Gaussian distribution is not practically possible as negative values of pore radius cannot exist. The log normal distribution was first introduced by Michaels [33]. A monomodal homoporous, bimodal pore size distribution to determine sieving coefficients was suggested by Wendt et al. [34-35]. Kassotis et al. [36] assumed that solute rejection was governed by geometric considerations only and suggested a mathematical model relating the flux and solute rejection to the pore size distribution function and the total number of pores. Ren et al. [37] have recently suggested a new model to determine the pore size distribution and the NMWL. However the pore size distribution cannot be determined precisely from a single probability correlation function [38]. The log normal distribution is still the most widely used for pore size distribution of ultrafiltration membranes.

In this paper we have modeled the pore size distribution of 300, 1000 kDa regenerated cellulose (RC) and 100, 300 kDa poly ether sulfone (PE) membranes. To determine the pore size distribution, ten SEM images were collected for each membrane. The pores in the image were analyzed and characterized using image analysis functions in MATLAB and a discrete pore size distribution was generated. The discrete distribution was fit to a lognormal distribution based on pore radius. Dextran rejection profile was calculated for the log normal fit using the pore radii.

2. Experimental

2.1 FESEM Experiments

Membrane samples were prepared by treating with ethanol water solutions containing 25%, 50%, 75%, 100%, ethanol followed by supercritical drying using carbon dioxide to prevent the collapse of pores. The membrane samples were then attached with copper tape on top of aluminum stubs and sputtered with a gold layer, 5 nm thick using a Hummer VII sputtering system (Anatech Ltd., Alexandria, VA). The JEOL JSM-6500F Field Emission scanning electron microscope (Peabody, MA) at an accelerating voltage of 5kV was used to image the membrane surface.

To determine the thickness of the functional layer additional images were taken. Critical point drying was carried out in the manner described above. The dried membrane samples were frozen in liquid nitrogen and cross-sections were obtained by slicing the samples with a sharp blade.
These were then attached to the sides of the sample holder with copper tape and sputtered with gold, 5 nm thick prior to FESEM.

2.2 Modeling

Based on the Hagen Poiseuille approximation, we have assumed an ideal membrane having an array of cylindrical pores of various radii in parallel. The radially averaged solution velocity, v (m/s), through this ideal membrane is given by:

$$v = \frac{r^2 \Delta P}{8 \eta L}$$

where r is the pore radius (m), \( \Delta P \) is the pressure drop across the pore (Pa), \( \eta \) is the solution viscosity (Pa-sec) and L is the membrane functional layer thickness (m).

To generate the discrete pore size distribution ten FESEM images were analyzed. Image analysis functions in MATLAB were used to define the pore circumference in the image. For each pore in the image we calculated the equivalent diameter of a circle with an equal cross sectional area. A histogram with bin size 2 nm was constructed using a data set containing the equivalent diameters of all the pores in the images. The discrete pore distribution function (PDF) was then determined using the histograms and the total number of pores. The following form of the log normal distribution was used to fit the PDF to the pore radius discrete distribution (Belfort et al., 1993):

$$n(r) = \frac{n_0}{r \sqrt{2 \pi}} \left[ \ln \left( 1 + \left( \frac{\sigma^*}{r^*} \right)^2 \right) \right]^{-1/2} \exp \left( \frac{-\ln \left( \frac{r}{r^*} \right) - 2 \ln \left( 1 + \left( \frac{\sigma^*}{r^*} \right)^2 \right)}{2 \ln \left( 1 + \left( \frac{\sigma^*}{r^*} \right)^2 \right)} \right)$$

$$R_m = r^* \left( 1 + \left( \frac{\sigma^*}{r^*} \right)^2 \right)^{-3/2}$$

We have also obtained a calculated rejection curve by using literature correlations for the resistance to flow of a given molecular weight Dextran for a given pore radius. The rejection coefficient \( (R_k) \) for each species k at high Peclet numbers (Pe $\gg$ 1) when diffusion terms are neglected (46) is defined as:

$$R_k = 1 - W_k$$
where $W$ is the convective hinderance coefficient

The Peclet number, which is a dimensionless number is defined as the ratio of convection rate to diffusion rate and is used to determine the dominant mechanism.

$$Pe = \frac{WuL}{HD_w} \quad (6)$$

For our calculations we have assumed our ultrafiltration cell to be well mixed and thus negligible concentration polarization. Based on theoretical models (Bungay and Brunner, 1973), the convective and diffusion hinderance coefficients are calculated as:

$$W = \frac{\Phi(2-\Phi)K_s}{2K_T} \quad (7)$$
$$H = \frac{6\pi\Phi}{K_T} \quad (8)$$
$$\Phi = (1-\lambda)^2 \quad (9)$$
$$\lambda = \frac{r_h}{r_p} \quad (10)$$

$$K_s = \frac{9}{4} \pi^2 \sqrt{2(1-\lambda)^2} \left[ 1 + \sum_{n=1}^{4} a_n (1-\lambda)^n \right] + \sum_{n=0}^{4} (a_{n+3} \lambda_n \quad (11)$$

$$K_T = \frac{9}{4} \pi^2 \sqrt{2(1-\lambda)^2} \left[ 1 + \sum_{n=1}^{2} b_n (1-\lambda)^n \right] + \sum_{n=0}^{4} (b_{n+3} \lambda_n \quad (12)$$

Where $r_p$ is the pore radii and the coefficients for the hydrodynamic functions $K_s$ and $K_t$ are given in Table 3. The hydrodynamic radius, $r_h$ for each dextran fraction was obtained from available technical literature.

**Table 3: Coefficients for the hydrodynamic functions $K_s$ and $K_t$**

| n  | $a_n$           | $b_n$  
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>-73/60</td>
<td>7/60</td>
</tr>
<tr>
<td>2</td>
<td>77/293/50400</td>
<td>-2227/50400</td>
</tr>
<tr>
<td>3</td>
<td>-22.5083</td>
<td>4.0180</td>
</tr>
<tr>
<td>4</td>
<td>-5.6117</td>
<td>-3.9788</td>
</tr>
<tr>
<td>5</td>
<td>-0.3363</td>
<td>-1.9215</td>
</tr>
<tr>
<td>6</td>
<td>-1.216</td>
<td>4.392</td>
</tr>
<tr>
<td>7</td>
<td>1.647</td>
<td>5.006</td>
</tr>
</tbody>
</table>

**3. Results**

3.3 FESEM Imaging

FESEM images of the membrane surfaces obtained at a magnification of 50000 are shown in figures 1. A membrane pore size distribution was obtained from image analysis. The membrane characteristics determined from surface analysis are outlined in table 3.
The mean pore radius from FESEM analysis is nearly same for all the four membranes, however the NMWCO vary due the presence of a pore size distribution. The PES 300 membrane has a larger number of pores with radii greater than 4 nm as compared to RC 300.

<table>
<thead>
<tr>
<th>Membrane</th>
<th>From Analysis</th>
<th>From FESEM</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean Pore radii (nm)</td>
<td>Smallest Pore radii (nm)</td>
</tr>
<tr>
<td>RC 300</td>
<td>4</td>
<td>1</td>
</tr>
<tr>
<td>RC 1000</td>
<td>2.5</td>
<td>1</td>
</tr>
<tr>
<td>PES 100</td>
<td>3</td>
<td>1</td>
</tr>
<tr>
<td>PES 300</td>
<td>4</td>
<td>1</td>
</tr>
</tbody>
</table>

Table 3: Membrane characteristics from FESEM analysis

3.4 Modeling results

To model the rejection, determination of Peclet number and consequently the flow through the membrane needs to be determined. For this purpose the thickness of the functional layer was determined from FESEM images, shown in figures 2. The RC 300 and 1000 kDa membranes had average functional layer thicknesses of 6 and 14 μm respectively. The average values were 85 and 70 μm for PES 100 and 300 kDa respectively.

The log-normal distribution obtained was then fitted to the discrete pore size distribution obtained from image analysis. The discrete and the fitted distributions are shown in figures 3-6. The various parameter values of the log-normal distributions used to fit the discrete pore size distribution are tabulated in table 4.

<table>
<thead>
<tr>
<th>Membrane</th>
<th>σ*(nm)</th>
<th>r*(nm)</th>
<th>Rm(nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>RC 300</td>
<td>1.99</td>
<td>5</td>
<td>4</td>
</tr>
<tr>
<td>RC 1000</td>
<td>2.2</td>
<td>3.6</td>
<td>2.236</td>
</tr>
<tr>
<td>PES 100</td>
<td>1.35</td>
<td>3.639</td>
<td>3</td>
</tr>
<tr>
<td>PES 300</td>
<td>3</td>
<td>6</td>
<td>4.293</td>
</tr>
</tbody>
</table>

Table 4: Log-normal distribution parameters

We do not claim that the membranes actually have a log-normal pore size distribution with the aforementioned parameter values. These parameter values only indicate the fact that the membrane pore size distributions for the membranes used in our study can be modeled using a log-normal distribution with the above tabulated values of σ* and r*. The rejection was calculated according to the equations outlined earlier and is shown in figures 7. The modeled rejection curves are S-shaped and predict a certain amount of rejection of solutes having hydrodynamic radii less than the pore radii. This is due to the inherent assumptions of the model.
Fig 1: FESEM images of membrane surfaces, a) RC 300, b) RC1000, c) PES 100, d) PES 300

Fig 2: Functional layer thickness, a) RC 300, b)RC 1000, c) PES 100, d) PES 300

Fig 3: Discrete and Log-normal distribution functions for RC 300
Fig 4: Discrete and Log-normal distribution functions for RC 1000

Fig 5: Discrete and Log-normal distribution functions for PES 100

Fig 6: Discrete and Log-normal distribution functions for PES 300
Fig 7: Modeled Rejection for RC 300, RC1000, PES 100 and PES 300

References

10. Calvo, J.I.; Pradanos, P.; Hernandez, A.; Bowen, W.R.; Hilal, N.; Lovitt, R.W.; Williams, P.M.; Bulk and surface characterization of composite UF membranes atomic force


ABSTRACT

Remediation feasibility studies are being conducted with mesocosm experiments for tetrachloroethene (PCE) contaminated water. A chamber is divided into six channels and filled with soil, and plants are grown on top. Contaminated water was fed at the bottom of each channel and collected at a bottom outlet, simulating groundwater flow conditions. Starting March 12, 2004, PCE was introduced at a concentration of about 2 mg/L (~12 μmoles/L) in three channels, two of them with alfalfa plants and the other with fescue grass. After the system attained steady state, the concentrations of PCE at inlet and outlet were monitored and the amount of PCE disappearing in the channel was measured. No degradation products were found at the outlet after about 100 days. Hence one channel with alfalfa was made anaerobic by adding 2 g of glucose in an aqueous solution. The glucose solution was fed starting July 1, 2004 with twelve doses injected over a 30-month period. From October 1, 2004, one liter of 0.1% emulsified soy oil methyl esters (SOME) was fed to another channel with alfalfa. The SOME was injected on thirteen occasions over a 27-month period. Most of the inlet PCE was converted to dichloroethene (DCE) in both channels. Three doses of KB-1, a consortium of Dehalococcoides spp, were injected into the channels on three different dates. After the introduction of KB-1, DCE was degraded (as measured at the outlet water) to low levels (< 5%) in the glucose fed channel, but ~40% of DCE still remained at the outlet of SOME fed channel. The outlet solution of the third channel (grass grown on top) in which no supplements were added, showed no degradation compounds. These results were used in implementing a biostimulation/bioaugmentation pilot study project for a PCE contaminated site in Manhattan, Kansas.

INTRODUCTION

Chlorinated solvents have been widely used as degreasers in various industries and as fumigants in grain storage facilities. The largest use for tetrachloroethylene or perchloroethene (PCE) is in dry cleaning and textile operations, accounting for an estimated 60 percent of all PCE use in the US in 1991. Dry cleaning chlorinated solvents are the second most ubiquitous contaminants, next to petroleum hydrocarbons. In the United States, soil and groundwater at approximately 400,000 sites are contaminated with chlorinated solvents (Sutfin, J.A. 1996).

Bioremediation, both natural and enhanced has proven to be a powerful approach for remediating chlorinated solvents, including PCE. In recent years, it has become apparent that biologically mediated degradation mechanisms may be important for chlorinated solvents. The degradation rate of PCE and its intermediate compounds, vary depending on the specific microorganisms and the nutrients present in the site. Usually, the rate limiting step is the degradation of cis 1,2-DCE to vinyl chloride (Nakashima et al., 2002).

Bioaugmentation with cultures containing Dehalococcoides is an effective means of remediating chlorinated ethenes at contaminated sites where halorespiring organisms are not naturally present or abundant (Ellis et al, 2000; Major et al, 2002). Fermenters transform electron-donating substrates to hydrogen and acetate, which are then used by Dehalococcoides, often regarded as the primary halorespiring population. Sulfate-reducers, acetogens, and
methanogens are competitors for available hydrogen. Yet perhaps these other populations assist in the dechlorination process, either directly or by providing essential micronutrients to halorespiring species.

KB-1, a consortium of *Dehalococcoides spp*, has been proven to be effective dehalogenators, both in laboratory (Ibbini et al, 2006) and field (Major et al, 2002). These microbes are strictly anaerobic and require conditions such as dissolved oxygen (DO) < 0.2 mg/L and redox potential < -50 mV. KB-1 was purchased from SiREM, Ontario, Canada.

In this work, the degradation of tetrachloroethene (PCE) was studied in a six-channel soil column system. The degradation of PCE is mainly limited by the availability of electron donors. Hence, two different substrates were used in this study, to create the necessary reducing conditions favorable for PCE transformation.

EXPERIMENTAL SYSTEM AND METHODS

The experimental system consisted of six independent channels that were constructed with steel bottoms, side panels, and end panels. Each channel was 110 cm long, 10 cm wide and 65 cm high with soil depth of 60 cm. The channels were packed with alluvial silty sand soil (< 10% silt) collected near a landfill in Riley County, Kansas. This soil system was used for contaminant fate studies previously (Zhang, 1999). Channels 1, 2 & 3 were used for the PCE study. The channels were filled with soil (up to 60 cm), and alfalfa was grown in channels 1 and 2, while fescue grass was grown in channel 3. A pair of fluorescent tube lights (40 W) at a height of 50 cm above the soil, for each channel, provides the light source for the plants. The inlet water was fed at 5 cm above the bottom of the channels (Figure 1). Watering of plants started from November 5, 2003. During the initial period the plants were observed, without contaminants in the soil. Until March 12, 2004, only distilled water was fed into the channels and the plants were growing well.

The contaminant was introduced starting from March 12, 2004. PCE was introduced at a concentration of about 2 mg/L (~12 µmoles/L) in three channels, two of them with alfalfa plants and the other with fescue grass. The height of saturated zone in each channel was controlled by the vertical position of the end of each outlet tube (25 cm in this system). Plants were harvested at the beginning of each month by cutting the top portion; after harvest, the plant height was approximately 10 cm. At the end of the month, the fescue grass and alfalfa grew to a height of approximately 50 cm and 70 cm, respectively.

After the initial preparation of inlet contaminant solution, 100 mL of ~40 mg/L PCE stock solution (prepared from PCE 99% purity, Sigma-Aldrich, St. Louis, MO)) and 900 mL of distilled water were added everyday. This stock solution was necessary to maintain approximately 2 mg/L in the inlet solution to each channel. As the inlet solution flows out of the bottle, the volume of the headspace in the inlet bottle increases, and air is drawn into the inlet bottle through a needle provided at the top of the inlet bottle. Approximately one L of air enters the inlet bottle in the course of a day and the PCE concentration in the headspace is maintained by mass transfer of PCE from the liquid phase. The dimensionless Henry’s constant of PCE at room temperature (25°C) is 0.72. There is a loss of PCE while adding 1 L of water and contaminant solution the next day because one liter of gas phase leaves the inlet bottle. Even though plants were grown in the system, the study primarily focused on the saturated zone of the channels and not on rhizosphere effects. The plants served as a natural pump, evapotranspiring some of the contaminated water and thus, influencing the residence time of the compounds in the channels.
There were five monitoring wells containing sintered alumina with polyethylene tubing for channel 1 and glass tubes with fritted glass at the bottom along the length of channel 2 through which groundwater samples could be collected from near the bottom of the channel (see Figure 1). In channel 1, the wells were placed at a distance of 13, 35, 60, 75 and 100 cm from the inlet. In channel 2, the wells were placed at a distance of 12, 32, 59, 77 and 100 cm from the inlet.

Inlet/Outlet Analysis
After introducing the contaminant solution on March 12, 2004, the concentrations of the contaminants were analysed at the inlet and the outlet of the channels. Ten mL samples were collected from the inlet tube at the entrance to each channel and from the outlet of each channel. Samples were collected using a 10 mL syringe and transferred to a 25 mL glass vial (22 mL nominal volume, Supelco, Bellefonte, PA) and closed with a mininert cap immediately. The vials were shaken manually and the compounds in the sample were allowed to partition into the headspace and attain equilibrium concentrations. The headspace samples were analysed with a gas chromatograph (GC).

Biostimulation by Glucose, Corn Starch and Soy Oil Methyl Esters
Because no degradation was observed after 100 days of introduction of the contaminant, supplements were added to stimulate growth of indigenous microbes, to create anaerobic
conditions, and also to supply hydrogen by fermentation. Introduction of 100 mL of 2% (w/w) glucose solution (Dextrose, anhydrous, Fisher Chemicals, Fairlawn, NJ) as an electron donor into channel 2 (alfalfa grown on top) resulted in anaerobic conditions in the channel. The glucose solution was fed once every month starting from June 30, 2004 and continued until February 2005 and intermittently on several occasions until January 1, 2007. From October 2004 (dose 4), the same mass of glucose (2 g but in 1000 mL) was fed over a longer period of time (approximately 1 day) to distribute it more efficiently. Totally, 12 doses of glucose solution were added. Corn starch was fed instead of glucose on days 266, 299, 328 and 522. From October 1, 2004, one liter of 0.1% emulsified soy oil methyl esters (SOME) was fed to channel 1 (alfalfa grown on top). Later SOME was sometimes injected in the wells instead of the inlet, since it tends to stay at the inlet due to its negligible solubility in water (SoyGold MSDS, 1998). When injected directly into the wells, 100 mL of 1% SOME solution was used.

**Bioaugmentation with KB-1**

Since the degradation of PCE stopped at the stage of DCE in the six-channel system, it was concluded that the native microbes were not able to degrade DCE. Hence, KB-1, a consortium of *Dehalococcoides spp.*, was added to channels 1 and 2. Since KB-1 is strictly anaerobic, it has to be injected in an oxygen free manner and the channels were operated to maintain that condition. For this purpose, it was planned to inject some amount of anaerobic water before injecting KB-1 and also to chase the KB-1 with the anaerobic water. The water for this purpose was obtained from the inlet of the channels. When the inlet tube of the channel was pulled out, the solution from the channel drips out. Another tube, with same diameter as inlet tube, is connected to the inlet of the channel and 300 mL of solution from near the inlet of the channel was collected in a 300 mL amber bottle while flushing with nitrogen gas, to maintain it oxygen free. One gm of glucose was then added to this solution and used as anaerobic water. Before adding KB-1, 100 mL of this solution is injected at the inlet. Fifty mL of this solution was taken in a 50 mL syringe and 2 mL of KB-1 culture was then injected into the syringe, thus mixing the culture with 50 mL of solution. This solution was then injected into the inlet of the channel and then chased with 150 mL of the glucose solution. The inlet tube from the inlet contaminant reservoir is then reconnected to the inlet of the channel.

**Analytical Methods**

Concentrations of chlorinated compounds and methane were measured using a gas chromatograph (HP 5890 Series II, Wilmington, DE) equipped with a Flame Ionization Detector (FID) and a HP-1 column (Dimethyl Polysiloxane matrix, 30 m x 0.53 mm, Agilent Technologies, Wilmington, DE). Hydrogen was the carrier gas. The injector temperature was set at 200°C and detector temperature was set at 300°C. Sample volume of 100 µL was injected in the column, which was at 100°C, and run for 5 minutes.

**RESULTS AND DISCUSSION**

**Inlet/Outlet Analysis**

Figures 2, 3 and 4 show the inlet PCE, outlet PCE and degradation product concentrations for glucose/corn starch fed channel, SOME fed channel and control channel respectively. In Figures 2a and 3a, a solid line is used to show the concentration of the sum of the chlorinated ethenes (CEs).
Glucose/corn starch fed channel

Forty days after first feeding glucose solution, the outlet PCE concentration started to decrease and eventually reached a low concentration (< 1 μmoles/L) in 3 months (Figure 2a). Correspondingly, the concentrations of cis 1,2-DCE also increased in the outlet liquid. For an inlet concentration of about 12 μmoles PCE/L, the outlet water had concentrations of about 10 μmoles/L of cis 1,2-DCE and methane concentrations ranging up to 135 μmoles/L. A mass balance of the inlet and outlet liquid revealed that the methane (Figure 2b) in the outlet water could have been generated from the glucose added.

Glucose addition led to conversion of PCE mainly to DCE and not other degradation products (Figure 2a). Breakdown of DCE is usually the rate limiting step in the degradation of PCE (Major et al, 2002). With glucose, almost the entire inlet PCE is converted to DCE; however with SOME, more of the entering PCE exits in the outlet (Figure 3a). Since SOME is sparingly soluble in water, the microbial degradation may be limited by availability of SOME in

![Graph](image)

**Figure 2a.** Inlet PCE and outlet PCE and DCE concentrations for channel 2. Glucose solution was added (indicated by + symbols) on day 110, 151, 173, 203, 236, 364, 676, 799, 901, 955, 994 (well 1) & 1025 (well 2). Corn starch was used on days 266, 299 328 & 522. KB-1 was injected on days 591, 810 and 958 (well 3).
Figure 2b. Outlet TCE and methane concentrations for channel 2. See Figure 2a for glucose and KB-1 additions.

Figure 3a. Inlet PCE and outlet PCE, DCE, and total CEs concentrations for channel 1. Water samples taken on indicated days after beginning (March 12, 2004) exposure. Soy Oil Esters were added (indicated by + symbols) on days 203, 236, 266, 299, 328, 359, 387, 415, 417 (well 3), 594, 954 (well 3), 991 (well 3) & 1022 (well 1); 100 mL of 1% SOME was used for injection into wells. KB-1 was added on day 605, 811 and 958 (well 3).
Figure 3b. Outlet TCE, VC & methane concentrations (right hand y-axis) for channel 1. Water samples taken on indicated days after beginning (March 12, 2004) exposure. See Figure 3a for times of SOME and KB-1 additions.

Figure 4. PCE concentrations in inlet and outlet of channel 3. Water samples taken on indicated days after beginning exposure (March 12, 2004).

the later portion of the channel downgradient to well 1. Different microbial populations are likely supported by the supplements, glucose and SOME. The glucose feeding was stopped after day 328, February 3, 2005. After day 400, concentrations of outlet PCE and TCE started to increase and DCE decreased. Outlet PCE in channel 2 increased and by day 437, it had reached 30% of
inlet value. However, even by day 475 (~150 days after stopping glucose feeding) PCE degradation was observed. Mass balance calculations and stoichiometric requirements indicate that one dose of glucose solution is sufficient for about 8 months of PCE entering the channel. More than 190 days after the last dose of glucose, PCE degradation was taking place even though the extent of conversion was dropping gradually with appearance of TCE (Figure 2b) also at the outlet between days 437 to 551. The food added was converted to biomass and when the channel runs out of food, endogenous decay may also come into play and provide the carbon and energy required for the biomass. Glucose may be converted into other compounds and stored; this may slowly be released and supply electron donor for PCE degradation. PCE concentration in the outlet increased but did not reach the inlet value. The inlet PCE is ~12 μM which is converted to about 3 μM TCE, 8 μM DCE and remaining 1 μM flows out.

Glucose solution was again added on several days as shown in Figure 2a. After the addition of glucose on day 522 and day 564, the outlet PCE and TCE concentrations started to decrease. By day 551, DCE concentration increased and reached the value of inlet PCE concentration by day 579. KB-1 was injected at the inlet on day 591 but it did not have any immediate significant effect. This culture of KB-1 was stored in the refrigerator for more than a year and therefore, may not have been active.

There was an increase in PCE and decrease in DCE around days 650 and 750, due to depletion of carbon and energy source. Glucose solution was added whenever the above happened, to maintain the outlet PCE concentration at low levels. KB-1 was again added at the inlet on day 810 resulting in a decrease of DCE by more than 50%. The concentration of DCE was maintained between 3.3 μM and 8.8 μM between days 810 to 948. KB-1 may not be dispersed throughout the length of the channel. KB-1 migrates very slowly in soils (Major et al, 2002) and spreads in the soil by growth of KB-1 biomass. After reduction of DCE to ~50%, KB-1 and/or energy source may not be available in the rest of the flow path. Hence, KB-1 culture was introduced into well 3, halfway along the channel, on day 958. Glucose solution was also added on days 994 and 1025 into well 1 and well 2, respectively, instead of at the inlet. After day 994, DCE concentration reached low levels and then was maintained below 1.5 μM until the end of the experiment (day 1113).

SOME fed Channel

From October 1, 2004, channel 1 was fed with one liter of 0.1 % emulsified soy oil methyl esters (SOME). The outlet liquid of the channel fed with PCE and SOME started to show some of the degradation compounds of PCE; however, the lag time for starting PCE degradation was longer compared to the glucose fed channel. This could be due to availability of SOME to the microbes along the length of the channel; SOME, being hydrophobic may stay at the inlet, the point of injection, in contrast to the glucose solution which is distributed throughout the channel.

In channel 1, the outlet PCE concentration decreased after the addition of SOME (Figure 3a) with conversion of PCE to TCE, DCE, VC and methane. DCE and TCE in the outlet increased with treatment time. Maximum concentrations reached were 2.8 μM TCE, 17 μM DCE, 1.2 μM VC and 212 μM methane. SOME solution was added monthly in channel 1, and the low outlet PCE concentration with high DCE concentration in the outlet was observed to continue.

SOME solution was added in channel 1 inlet on May 1, 2005 (day 415). Since the entire inlet PCE was not transformed in channel 1, it is possible that the SOME fed at the inlet could be trapped in the initial portion of the channel. On May 3, 2005 (day 417), in addition to the SOME
fed at the inlet, 100 mL of 1% SOME solution was directly injected into well 3, at a distance of 60 cm from inlet. This addition had a significant effect on the PCE and other product concentrations at the outlet as shown in Figures 3a and 3b. After SOME solution was added on May 3, 2005 (day 417), feeding of SOME was stopped to study the capability of the channel to carry on PCE degradation with the electron donor stored over the past eight months. In the period from day 411 to day 475, the outlet concentration of PCE decreased from about 5 µM to 0.2 µM, TCE decreased from about 2.5 µM to 0.1 µM, DCE increased from about 5 µM to 17 µM and methane increased from about 0.5 µM to 9 µM (Figures 3a and 3b). SOME solution was added on nine occasions prior to day 417 and the tenth dose was on day 594. With this SOME, the microorganisms were capable of degrading PCE until day 954, i.e., for 360 days. Since SOME does not dissolve appreciably in water, it appears to be retained in the channel longer than a soluble substrate. A portion of the food added was converted to biomass and when the channel runs out of food, endogenous decay may also come into play and provide the carbon and energy required for the biomass. SOME solution was added again on days 991 and day 1022 to maintain low redox condition in the channel, which was conducive to KB-1. The outlet solution had nearly equimolar amounts of DCE as inlet PCE, and the native microbes were not capable of degrading DCE further. To degrade DCE, KB-1 was added at the inlet on day 605 and day 811. This addition did not have significant effect on the outlet DCE. SOME and KB-1 may stay at the inlet and not be available in the rest of the channel length to degrade DCE. Based on this hypothesis, KB-1 was added into well 3 (60 cm from inlet of channel) on day 958. This addition had a significant effect: By day 994 DCE decreased to 7.9 µM from about 16 µM on day 975, and it continued to decrease and reached a value of 5.8 µM by day 1113. KB-1 was able to degrade DCE with both SOME and glucose supplements, however, with glucose, DCE concentration reached very low values such as < 1 µM.

Control channel

The outlet solution of the channel (grass grown on top) in which no energy supplements were added, showed no degradation compounds of PCE (Figure 4). The outlet PCE concentration was almost the same as inlet PCE concentration. Based on the study of these three channels, it can be concluded that the PCE degradation is certainly an electron-donor-limited process, which benefits from adding supplements such as glucose and SOME.

CONCLUSIONS

Supplements such as glucose, corn starch, and SOME stimulated the indigenous microbes and helped in the degradation of tetrachloroethene (PCE). The pattern and rate of degradation of PCE varied with different supplements. The degradation compound ratios were not the same in the glucose/corn starch and SOME amended channels. Glucose was found to be a better supplement than SOME with respect to the onset time for PCE degradation. Because glucose is soluble in water, it is distributed more rapidly.

Most of the degradation process took place in the initial portion of the SOME fed channel, since SOME likely stayed near the inlet of the channel, due to sorption and retarded flow. SOME is a NAPL which does not flow freely like an aqueous solution. It may also sorb to soil organic matter in the initial portion of the channel. This study demonstrated that the supplements glucose and SOME are effective substrates that can be added to PCE contaminated groundwater to promote degradation.

The mesocosm studies have shown that there is a significant residual effect of introduced carbon supplements. Mass balance and stoichiometric requirements of substrates for degradation
of PCE suggest that about 10% of the nutrient supplements glucose and SOME are used to consume the oxygen in the inlet water. In the soil system, the microbes, which are present as a biofilm, adsorb glucose and SOME, store polysaccharides, and have a reservoir of carbohydrates to use to reduce PCE to DCE. There is also the effect of distributed flow and channeling. These phenomena influence the length of time the system operates effectively and degrades PCE after food is added. Because SOME has very low solubility in water, it is retained near the point of injection, and it is able to provide needed substrate for a relatively long time compared to a soluble substrate such as glucose.

Addition of KB-1 at well 3 had significant impact in the degradation of DCE, in both glucose and SOME amended channels, compared to addition at the inlet. KB-1 added on day 958 at well 3 was active even on day 1113 (155 days) suggesting that there is sustainable growth of KB-1 when provided with suitable conditions and substrates. The well sample results impart an idea of how SOME should be applied in the field: it should be injected at several points down-gradient rather than at a single point.

ACKNOWLEDGEMENT
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Emergency Preparation and Green Engineering

1,2 Clinton Whiteley, 1 Terrie Boguski, and 1,2 Larry Erickson, 1 Ryan Green

1 Center for Hazardous Substance Research, 104 Ward Hall, Kansas State University, Manhattan, KS 66506; 2 Department of Chemical Engineering, 105 Durland Hall, Kansas State University, Manhattan, KS 66506

Abstract
As our society continues to better prepare itself for biological, radiological, and chemical emergencies, there is a need for better and more readily available emergency planning information for program managers and military/business personnel. An online hazardous materials and emergency planning tool for the Environmental Knowledge and Assessment Tool (EKAT: www.ekat-tool.com) could fill that need.

The online emergency preparation and green engineering tool would be one that could provide the user with information regarding hazardous materials training, links to local emergency response teams and resources, online guides for emergency planning, and scenarios to illustrate HAZMAT situations that the user may face at military installations or other places. As greater emphasis is placed on sustainability, there is a growing interest in information giving users the ability to evaluated the "greenness" of their processes. To have a tool that will not only guide one during HAZMAT emergencies, but also help him or her evaluate how chemicals affect health and environment, would be invaluable.

Introduction:
EKAT is an automated tool created to identify, research, and evaluate environmental and safety-related issues for products and systems. The program presents basic information on technical and regulatory requirements and serves as a preliminary environmental screening tool for potential issues of concern. EKAT also serves as a resource center, linking to other references, tools, and databases to assist in research efforts to minimize any unintentional safety and environmental effects associated with product or system use.

While content within EKAT begins to address emergency planning and response needs of the current user, a multi-faceted, interactive education tool on this topic would directly benefit the Marine Corps as well as other potential users. To have a tool that will not only guide one during a specific HAZMAT emergency, but also help prepare for response to a potential disaster, would be invaluable. The work describes the present vision for the Emergency Preparation and Green Engineering (EPGE) tool, which is being developed.

The idea for this tool stems from the need for better and more rapid availability of emergency planning information for a program manager or installation/business environmental health and safety personnel. Emergency information is available on the Web, but accessing it and getting all the information that one needs can be time consuming. The Emergency Preparation and
Green Engineering (EPGE) tool could be an excellent resource to have in hazardous emergency response situations. The tool will include but is not limited to, four sections; Contacts Database, Written Report Generator, HAZWOPER Training Resource, and a Green engineering section; see Figure 1.

Figure 1: Example page layout for the EPGE tool.

Overview:
The emergency preparation and green engineering tool will not only aid the user in generating reports and screening materials, but will also provide an on-line training tool, a database of important emergency information, and a green engineering section. The tool’s main components can be organized in the following manner.

1. **Report Generator**
   According to the OSHAct of 1970, “Your employer must make available information on safety and health hazards in the work area, precautions to be taken, and procedures to be followed in the event of an accident or exposure to toxic substance.” This means that every company must have available written emergency plans. The Emergency Preparation and Green Engineering (EPGE) tool could aid the user in creating, organizing, and quickly referencing this multitude of written plans or reports, which would make the process more efficient and possibly produce better results. In addition to providing valuable information to the current EKAT client, this tool could enable EKAT to better serve another market of available users that includes businesses and local/state governments and response teams that are responsible for handling hazardous materials.

As an initial working concept, the assessment tool would work much like the current generators do that are already in EKAT. The tool would provide a listing of reports that are commonly needed for hazardous materials and emergency situations, see Figure 2 for an
example template. The user may be able to simply print out existing report templates, or EKAT could also be designed to help guide the user in adding site specific information to generalized reports to make the reports specific to a facility or project. This will allow the company to bypass many hours of research and meetings. Often these reports are contracted out to other businesses. This can lead to extra expenses because of the involvement of the second party. However, if EKAT could provide the most up-to-date information in template form, it might save the user money and time.

**Electrical Work Plan**

<table>
<thead>
<tr>
<th>Company Name</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Job Name:</strong></td>
</tr>
<tr>
<td><strong>Location:</strong></td>
</tr>
<tr>
<td><strong>Effective Dates of Plan</strong></td>
</tr>
<tr>
<td>Start: End:</td>
</tr>
<tr>
<td><strong>Work To Be Performed by</strong></td>
</tr>
<tr>
<td><strong>Person in Charge:</strong></td>
</tr>
<tr>
<td><strong>Phone:</strong></td>
</tr>
<tr>
<td><strong>Description of Work:</strong></td>
</tr>
<tr>
<td><strong>Associated Hazards (e.g. voltage, current, etc.):</strong></td>
</tr>
<tr>
<td><strong>Hazard Mitigation (e.g. specific PPE, barriers, etc):</strong></td>
</tr>
<tr>
<td><strong>Inspection Required after Work? Yes No (Required for New Installations.)</strong></td>
</tr>
<tr>
<td><strong>Originator: Date:</strong></td>
</tr>
</tbody>
</table>

Figure 2: Example written report template for electrical work.

2. **Training Resource**

   Following and expanding upon the initial on-line training components currently found in EKAT, a second integral part of the tool would be the creation of tutorial lessons on various emergency planning, response, and hazardous materials topics. The training tool section is included because of the need for information availability. Comprehensive resource allows users to stay up-to-date on emergency procedures, as well as gives them the opportunity to learn more about a specific topic. In addition, this section could give information related to training courses, training locations, and instructor contact information.

   The training tool section will include a HAZWOPER resource text book, mock scenarios, and links to other outside training tools. The text book, though not a standard throughout the nation, is certified by OSHA. It will be organized to allow the user to link to sections of the
book; see Figure 3. This will grant them the ability to find the information that they want without having to search through the entire text.

The mock scenarios will include various examples of emergencies in PDF or WORD format. The user will be able to read the procedures that should be followed in each situation. In this way they will be able to think through a variety of scenarios, and better prepare themselves for a future emergency. The links to other outside training tools are provided because the EKAT team realizes that in the business environment there is a need for other training in addition to the HAZWOPER certification course.

Figure 3: Example format for the Training Resource page.

Emergency Database

The Emergency Database is in place for the situation when there is an actual emergency and the user needs to know what to do. In this case, the user would probably use some combination of all three sections of the hazardous materials and emergency planning tool. This section will provide specific contact information for regional HAZMAT and emergency response teams.

The types of contacts that will be included will be the regional HAZMAT teams, emergency response teams, state Fire Marshall's network, federal agencies, and the CHEMTREC Call Center. The regional HAZMAT teams contact information was acquired from the Federal Motor Carrier Safety Administration (FMCSA) State/Regional Hazardous Materials Contacts. This database offers not only the four regional contact numbers, but also phone numbers for all the individual states.
The Fire Marshall’s network is included because in general the Fire Marshall in the area is the person that oversees the emergency response project. Although the Fire Marshalls are in charge, it is also required to contact the federal agencies for most hazardous emergencies.

The CHEMTREC call center is a hotline for fire fighters, law enforcement, and other emergency responders to obtain critical information and assistance for emergency incidents involving chemicals and hazardous materials. CHEMTREC’s center is linked to the largest network of chemical and hazardous material experts in the world including response specialists within the carrier community, public emergency services, and private contractors. When necessary, Environmental Support Solutions (ESS) can establish direct communications between these experts, CHEMTREC personnel, and the responders at the scene of an incident.

Along with the contact information the Emergency database will include a section that is related to the Department of Transportation (DOT) guide book and the Wireless Information System for Emergency Response (WISER). WISER is a system designed to assist first responders in hazardous material incidents. WISER provides a wide range of information on hazardous substances, including substance identification support, physical characteristics, human health information, and containment and suppression advice. This part of the database will give the user information on correct protective equipment and physical information about a chemical. It will also involve an “Unknown Chemical” identifier. This feature is already in use, with the WISER system, and the EKAT team has plans to adapt it and make it practical for their customers.

Green Engineering
The increase in interest in sustainability, environmental concerns and government regulations has inspired the chemical industry in both innovation and reformulation of products that address a broad spectrum of human and environmental health and safety factors. Defining “green” chemicals is difficult because the notion encompasses an array of factors: perception, empirical values, geographical area of use, the application for which they are used, available alternatives, and knowledge of the total manufacturing process.

Section four of the proposed tool is an environmental management/minimization component. Instead of just examining what potential concerns the user may have with a particular material, the tool can help suggest alternative chemicals that are less toxic or ecologically hazardous. This section would take advantage of EKAT’s extensive chemical database along with existing chemical compatibility spreadsheets; Solvent Alternatives Guide (SAGE), Coating Alternatives Guide (CAGE), Handbook of Green Chemicals, and the WISER database.

SAGE is a comprehensive guide designed to provide pollution prevention information on solvent and process alternatives for parts cleaning and degreasing. The Coatings Guide contains several tools to help users identify low-volatile organic compound/hazardous air pollutant coatings that may serve as drop-in replacements for existing coating operations. To date, the Coatings Guide information base has focused on alternative coatings for plastic and metal substrates.
The Handbook of Green Chemicals describes approximately 7000 trade names that provide one or more of the following green attributes: biodegradable, environmentally safe/friendly, recyclable, hazardous air pollutants (HAPs)-free, low ozone-depleting/ non-ozone-depleting, volatile organic compounds (VOC)-compliant/low-VOC/VOC-free, low global warming, low vapor pressure chemicals, non-chloro fluoro carbon (CFC)/non-HCFC, significant new alternatives policy (SNAP), nonhazardous, superfund amendments and reauthorization act (SARA)- nonreportable, halogen-free, noncarcinogenic, and nontoxic. Inclusion of products in this book was the result of a two-step process: manufacturers were canvassed for products meeting criteria listed above; these products were then screened for identifiable green attributes.

To make this section realistic, the EKAT team plans to assign a “greenness factor” to each chemical. This score will be based on physical information, environmental information, and safety and health information. The physical information could include toxicity (permissible exposure limit (PEL), reference exposure limit (REL), immediately dangerous to life and health (IDLH)), density, vapor pressure, and solubility in water. These properties are the basic building blocks of a chemical. With this information a company could evaluate the general environment that must be maintained to support their chemicals safely.

Environmental properties are not as black and white as physical properties, but are just as important, especially in the developing global climate change situation that our society faces. These properties are important because they represent the effect that an industry’s chemicals and processes have on the environment. The environmental information could include maximum contaminant level (MCL) concentration in drinking water, stratospheric ozone depletion, and photochemical smog formation. This section is needed to give the user an idea of what to expect when using certain chemicals, and to give them a picture of the consequences involved in manufacturing their products.

A safety and health section can be included in the “greenness factor” to give a general representation of how chemicals will affect their employees and the plants and animals in the surrounding area. Some of the important properties include human carcinogens, microbial and viral infection, and mutagenic compounds.

Conclusion:
The emergency preparation and green engineering tool has the potential to become very beneficial, based on the wide range of information and resources that it can provide. Emergency response applications have the potential to be of considerable use to not only current EKAT clients, but other public and commercial users as well. With the introduction of the green engineering section, the user would be able to make educated decisions on whether or not to use more environmentally friendly chemicals. In addition to providing the user with valuable information, this tool will allow EKAT to fulfill a public need by offering a service that is not yet available online.

Acknowledgements
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emergency planning and green engineering tool to be primarily developed for the Marine Corps with the prospect of adding new users in the future.

Resources

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