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Roger G. Harrison
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Preface

The 34th Annual Biochemical Engineering Symposium was held at the University of Oklahoma on April 23, 2005. The objectives of the symposium were to provide (1) an opportunity for students to present and publish their work, and (2) a forum for informal discussion of bioengineering research being carried out at the participating universities.

Fifteen papers presented at the symposium are included in these proceedings. Because final publication usually takes place in refereed journals, the articles here are typically brief and often cover work in progress.

Those who attended the symposium are the following:

Iowa State University: Peter Reilly, Zhengrong Gu, Anthony Hill, Chandrika Mulakala, Luis Petersen, Katie Pfeiffer, Taran Shilling, Kevin Smith, Murali Subramanian, Maria del Pilar Torres-Gonzalez, Li Xu, Cheng Zhang

Kansas State University: Larry Erickson, Amit Apte, Donifan Barahona, Siegfredo Castro Diaz, Edward D’Souza, Amol More, Sathishkumar Santharam

Oklahoma State University: Yan Huang, Aparna Sarasam

South Dakota School of Mines and Technology: Patrick Gilcrease, Michael Green, Muralidhar Reddy Mallem, Katie Standish


Financial support for this symposium from the College of Engineering and the School of Chemical, Biological and Materials Engineering at the University of Oklahoma is gratefully acknowledged.

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Environmental knowledge and assessment of tool, Amit Apte, Edward D’Souza, Clinton Whiteley, Rachit Yadav, L. E. Erickson, Terrie Boguski, Angie Burgoon, Ryan Green, Leslie Jamka, Greg Norris, John Blair, and Jay Fredkin, Kansas State University

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Symposium program
Design of an Animal Waste Treatment System by Combination of Technologies

Sigifredo Castro(1), Larry E. Erickson(2), Alok Bhandari(3), Lawrence C. Davis(1), Dean Thomson(4), and Bill Junk(4).

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Abstract

Animal feeding operations (AFOs) pose a number of risks to water quality and public health, due to the large amount of animal manure and wastewater that they generate. Manure from AFOs is rich in nutrients such as nitrogen (N), phosphorus (P), potassium (K), secondary nutrients, and metals such as copper. If not properly treated, excess nutrients can lead to eutrophication of surrounding water sources. The waste from AFOs also has the potential to contribute suspended solids, pathogens, oxygen-demanding materials, and heavy metals to surface and ground waters. In addition, AFOs can be a source of atmospheric pollutants, particularly ammonia, via volatilization. Handling of this waste is currently performed through (a) storage in lagoons and basins and (b) management using composting facilities, constructed wetlands, and application of manure or runoff water to agricultural land. The effectiveness (load reduction) of these practices is highly variable and requires strict controls to prevent water pollution. As the size and number of AFOs grow, so does the need for environmentally sound and economically efficient management practices. This manuscript describes a new system to overcome the problems with current animal waste handling and disposal methods. The proposed system combines and takes advantage of existing technologies such as an anaerobic digester and an aeration tank, and the recently developed Anammox reactor. The goals of this process are to (1) reduce the nitrogen content of manure waste, (2) generate low-cost energy, (3) reduce the odors and atmospheric contaminants, and (4) improve the overall quality of the liquid waste.

INTRODUCTION

Animal Feeding Operations

As a result of industrialization, animal feeding operations (AFOs) have increased in number and size, along with higher animal densities on relatively small land areas. This close confinement and the now narrowed farm-urban interface, raise concerns over the potential environmental hazards posed by these large AFOs (Cole et al., 2000). Gases generated from manure and urine that contaminate the air include ammonia, carbon monoxide, hydrogen sulfide, methane, and volatile fatty acids. Water-associated contaminants include oxygen-demanding substances such as organic matter, nutrients that could lead to eutrophication of surrounding surface waters (e.g., nitrogen, phosphorus, and potassium), salts, metals, and sediments. Manure may also contain pathogens, hormones, and antibiotics depending on the feed, supplements, and medications given to the animals (USEPA, 2004).
With the development of the 1997 Clean Water Action Plan, the USDA and USEPA established the Comprehensive Nutrient Management Plan (CNMP) to minimize the environmental and public health impact from AFOs. The CNMP sets guidelines for the storage, transport, and treatment of manure wastewater and the subsequent nutrient management through land treatment practices (USEPA, 2004).

The most generalized management measures for wastewater from AFOs, consist of waste storage, waste treatment, and transfer or utilization of the treatment products (USEPA, 2004). Waste treatment is achieved through aerobic or anaerobic treatment lagoons and anaerobic digesters. In an on-site anaerobic digester, the waste is confined in an air-tight vessel such as a covered lagoon, and decomposed producing biogas (USEPA, 2004). The digesters present several advantages over lagoons, including better odor and fly control, reduction of ammonia emission to the atmosphere, elimination of pathogenic and parasitic organisms, potential use of biogas as a source of energy, low biomass production, increased fertilizer value of biosolids, destruction of most weed seeds, and less susceptibility to severe weather (Sanchez, et. al., 2005). The biosolids generated from either of the treatment options could be further treated through composting. Depending on the final composition of the liquid effluent, this can be directed to surface waters, or used for land application through surface spreading and irrigation (USEPA, 2004).

Swine waste

Anaerobic lagoons are the most common means to treat swine waste in the U.S. and have been preferred because of their relatively low cost (Cheng and Liu, 2002, Guan and Holley, 2003). Although the organic and solid contents in animal waste can be reduced by anaerobic treatment, the high nitrogen levels in animal waste remain practically unaffected (Bhandari and Xia, 2003, Ballester et. al., 1992). Lands where the lagoon-treated manure is sprayed are often over-saturated from surplus liquid and nutrients (Guan and Holley, 2003). The nitrogen content of animal waste could be about 100 times higher than the average nitrogen content of domestic waters. While the concentration of total nitrogen in domestic sewage varies from 20 to 85 mg/L (Sedlak, 1991), the total nitrogen content of animal waste as produced can be over 4,000 mg/L (MWPS, 1985). In terms of mass of N per unit mass of manure produced, swine manure contains one of the highest levels of total nitrogen among the wastewaters from AFOs. Although the waste nitrogen generation might vary among the different swine production units (Table No. 1), these facilities can release nitrogen at an average rate of 18 g N/day/45-kg hog (Overcash et al., 2000).

Table No. 1: Nitrogen waste generation in swine facilities (Overcash et al., 2000)

<table>
<thead>
<tr>
<th>Production system</th>
<th>N (g/day/45-kg hog)</th>
<th>N (%TS)</th>
<th>NH$_3$-N/TKN$^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Concrete slab facilities</td>
<td>9 - 32</td>
<td>3.4 - 10</td>
<td>0.21 - 0.93</td>
</tr>
<tr>
<td>Slotted floor-pit</td>
<td>10 - 26</td>
<td>4.4 - 9.8</td>
<td>0.21 - 0.53</td>
</tr>
<tr>
<td>Unspecified confinement housing</td>
<td>9 - 38</td>
<td>3.3 - 8.8</td>
<td>0.45 - 0.74</td>
</tr>
</tbody>
</table>

$^a$ TKN: total Kjeldahl nitrogen

During anaerobic treatment, the organic nitrogen present in residual proteins and nucleic acids in the manure is generally transformed to amino acids and urea by hydrolysis. Amino acids and urea are further transformed by either bacterial conversion to free ammonium (NH$_4^+$) by ammonification or in a lesser extent by incorporation into the
anaerobic bacterial cells for microbial growth. At high pH, the ammonium is volatilized as ammonia gas \((NH_3)\) by the reaction shown in equation [1]:

\[ NH_4^+ + OH^- \rightarrow NH_3(gas) + H_2O \]  \[1\]

Currently, approximately 80% of ammonia \((NH_3)\) emissions in the U.S. originate from livestock waste. The annual \(NH_3\) emission rate from anaerobic lagoons treating swine waste was estimated to be 2.4 kg/year/pig based on field tests at one swine lagoon in North Carolina (Doorn and Natschke, 2002). Ammonia affects the respiratory system, is an irritant to the eyes, skin, and mucous membranes, and is one of the major contributors for odor problems around swine facilities (Cole et al., 2000). At low pH, the ammonium remains in the digested wastewater, which could be spread on fields or reach surface waters. Under aerobic conditions, ammonium is converted to nitrite \((NO_2^-)\) and/or nitrate \((NO_3^-)\) by nitrification. Contamination of potable water and groundwater poses a serious risk for human and animal health, e.g., “blue baby” syndrome (Bhandari and Xia, 2003).

Nitrification (Figure 1) is an autotrophic and aerobic process that involves the oxidation of \(NH_4^+\) to \(NO_2^-\) and further to \(NO_3^-\) with molecular oxygen as the electron acceptor. The reaction consumes a large amount of oxygen, requiring 4.2 g of oxygen for each gram of ammonium nitrogen nitrified (Khin and Annachhatre, 2004).

\[
\begin{align*}
\text{Nitrification} & \\
& \begin{align*}
NH_4^+ + 1.5O_2 & \rightarrow NO_2^- + 2H^+ + 2H_2O \\
NO_2^- + 0.5O_2 & \rightarrow NO_3^- 
\end{align*} \quad \text{(Nitrosomonas)} \quad [2] \\
& \begin{align*}
NO_2^- + 0.5O_2 & \rightarrow NO_3^- 
\end{align*} \quad \text{(Nitrobacter)} \quad [3]
\end{align*}
\]

\[
\begin{align*}
\text{Denitrification} & \\
& \begin{align*}
2NO_3^- + 10H^+ + 10e^- & \rightarrow N_2 + 2OH^- + 4H_2O \\
2NO_2^- + 6H^+ + 6e^- & \rightarrow N_2 + 2OH^- + 2H_2O 
\end{align*} \quad \text{(Proteobacteria)} \quad [4]
\end{align*}
\]

Figure 1: Scheme of the nitrification / denitrification process for nitrogen removal in conventional wastewater treatment.

Denitrification (Figure 1) is an anoxic and heterotrophic process to achieve the reduction of \(NO_3^-\) to \(NO_2^-\) to further \(N_2\). This is accomplished with a variety of electron donors, including methanol, acetate, ethanol, lactate, and glucose. The requirement of organic carbon for denitrification is significant; for example, 2.47 g of methanol / g \(NO_3^-\)-N are required for complete denitrification (Khin and Annachhatre, 2004).

As nitrification and denitrification are carried out under different conditions and by different microorganisms, experience shows that these processes have to be separated in space or time. Wastewaters containing high nitrogen concentration (e.g., swine manure) would require large amounts of oxygen to achieve complete nitrification and large amounts of organic carbon added to achieve complete denitrification. Thus, conventional nitrogen removal processes would present economic limitations for complete reduction of the high nitrogen levels in wastewaters from AFOs.
Anaerobic ammonium oxidation

A process to convert ammonia directly to nitrogen gas has been recently developed. The anaerobic ammonium oxidation (Anammox) using nitrite as the electron acceptor was first predicted from thermodynamic estimations by the Austrian professor E. Broda (1977):

\[ \text{NH}_4^+ + \text{NO}_2^- \rightarrow \text{N}_2 + 2\text{H}_2\text{O} \quad \Delta G = -335 \text{ kJ/mol NH}_4 \quad [6] \]

The actual process where this reaction takes place was discovered and patented by Mulder et al. (1995) in a lab-scale anaerobic fluidized denitrifying bed treating effluent from a methanogenic fermentor. The work of van de Graaf et al. (1995) determined that nitrite was the preferred electron acceptor for the process (equation [6]) and the carbon source was carbon dioxide. Strous et al. (1998) estimated the Anammox stoichiometry based on mass balance over Anammox enrichment cultures, as presented in equation [7]. Nitrate could be produced from nitrite to generate reducing equivalents for CO₂ fixation.

\[ \text{NH}_4^+ + 1.31\text{NO}_2^- + 0.066\text{HCO}_3^- + 0.13\text{H}^+ \rightarrow \]
\[ \rightarrow 1.02\text{N}_2 + 0.26\text{NO}_3^- + 0.066\text{CH}_2\text{O}_{0.5}\text{N}_{0.15} + 2.03\text{H}_2\text{O} \quad [7] \]

Hydrazine (N₂H₄) and hydroxylamine (NH₂OH) are known to be intermediates of the Anammox process, with a possible metabolic pathway as presented in Figure 2 (van de Graaf et al., 1997).

\[ \begin{align*}
\text{NH}_4^+ & \quad \text{NH}_2\text{OH} \quad \text{NO}_2^- \quad \text{NO}_3^- \\
\text{N}_2\text{H}_4 & \quad 2[\text{H}] \quad \text{N}_2 \\
2[\text{H}] & \quad \text{[N}_2\text{H}_4] \\
& \quad \text{N}_2
\end{align*} \]

Figure 2: Possible metabolic pathway for the ANAMMOX reaction

The litho-autotrophic bacteria capable of the anaerobic oxidation of ammonia were identified as members of the order of Planctomycete (Strous et al., 1999a). Two microorganisms have been tentatively named as “Brocadia anammoxidans” (Strous et al., 1999a) and Kuenenia stuttgartiensis (Schmid et al., 2000). Some properties of Brocadia anammoxidans are shown in Table No. 2 (Jetten et al., 2002). Isolation of Anammox bacteria with conventional procedures has not been possible, because the activity only occurs when the bacteria are present in sufficiently high concentrations (> 10¹⁰ / mL). Nevertheless, their isolation was achieved by an optimized percoll density gradient centrifugation (Strous et al., 1999a).
Table No. 2: Properties of *Brocadia anammoxidans* (Jetten et al., 2002)

<table>
<thead>
<tr>
<th>Property</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phylogenic position</td>
<td>Planctomycetales</td>
</tr>
<tr>
<td>Morphological features</td>
<td>Coccoid, Proteinaceous S layer, crateriform structures, no peptidoglycan, internal compartment ‘anamoxosome’</td>
</tr>
<tr>
<td>Enzymes</td>
<td>Hydroxylamine oxidoreductase</td>
</tr>
<tr>
<td>Anaerobic rate</td>
<td>60 nmol / (mg protein) / min</td>
</tr>
<tr>
<td>Growth rate</td>
<td>0.003 / h</td>
</tr>
<tr>
<td>Doubling time</td>
<td>10.6 days</td>
</tr>
<tr>
<td>Growth yield</td>
<td>0.11 g VSS / g NH₄-N</td>
</tr>
<tr>
<td>Ks (ammonium)</td>
<td>5 µM</td>
</tr>
<tr>
<td>Ks (nitrite)</td>
<td>&lt; 5 µM</td>
</tr>
<tr>
<td>pH range</td>
<td>6.4 – 8.3</td>
</tr>
<tr>
<td>Temperature</td>
<td>20 - 43°C</td>
</tr>
</tbody>
</table>

Enrichment of Anammox bacterial cultures is particularly difficult, due to their extremely low growth rate and growth yields (Khin and Annachhatre, 2004). Under appropriate conditions, Anammox activity has been detected from 50 to 200 days after enrichment (Strous et al., 2002). Additionally, Anammox bacteria are sensitive to oxygen (as low as 2 µM), nitrite (between 5 and 10 mM), and phosphate (5 mM), resulting in reversible inhibition of the Anammox activity (Khin and Annachhatre, 2004). The presence of other microorganisms might also affect the successful enrichment of Anammox cultures by producing or consuming some of the reactants involved in the Anammox reaction.

Organic nitrogen compounds present in the feed can be released from bacterial lysis and be further transformed to ammonia by anaerobic ammonification. This might result in an increase of the ammonia concentration, which could mask any Anammox activity present (Ahn and Kim, 2004). Organic carbon present in the system could be used as electron donor by the anaerobic denitrifying bacteria, which would convert NO₃⁻ to N₂ leaving the ammonia intact. Finally, there are organisms capable of dissimilatory nitrate reduction to ammonia (DNRA), a process well recognized in anaerobic habitats such as the rumen, anaerobic sludge, and anoxic sediments (Tiedje, 1988). The DNRA involves two steps, the first one being the conventional nitrate respiration where NO₃⁻ is reduced to NO₂⁻. The latter is subsequently reduced to NH₄⁺ in the second step. The dissimilatory nitrate reduction to ammonia is regulated by oxygen and its main function is to act as an electron sink, i.e., when anaerobes are faced with a shortage of electron acceptors (Tiedje, 1988). Thus, the DNRA could interfere with the enrichment of Anammox bacteria by competing for the NO₂⁻ and producing more NH₄⁺.

Since Anammox bacteria grow in biofilms, enrichment cultures have been more successful in packed-bed and fluidized bed reactors (Strous et al., 2002). Some desirable characteristics of the reactor are good biomass retention, homogeneous distribution of substrates, reliable and stable long-term operation. These are necessary due to the low yield and long start-up times required to obtain sufficient biomass concentration of Anammox bacteria (Strous et al., 2002, Jetten et al., 2002).
PROPOSED ANIMAL WASTE TREATMENT SYSTEM

The objective of this study is to create an integrated system consisting of an anaerobic digester, an aeration tank, and an Anammox reactor for biological treatment of swine manure, as an alternative to anaerobic treatment lagoons and other strategies. The integrated system will economically overcome major environmental problems (odor, N removal) resulting from the limitations of conventional systems and will increase the overall efficiency. The proposed system is being investigated initially at a laboratory scale. The Anammox reactor is being explored first in order to obtain an enriched culture.

PRELIMINARY STUDIES WITH ANAMMox

Materials and methods

The enrichment culture for Anammox bacteria was set up in four reactors, each one consisting of a 500-mL Erlenmeyer flask with a side arm for gas exhaust (see Figure 3). The culture was performed statically in a water bath held a 35°C. The seed biomass selected was sludge from the anaerobic layer (hypolimnion) of a swine-waste treatment lagoon in a Pig Farm located in southwest Kansas (Keesecker). Prior experiments demonstrated that this sludge had potential for containing Anammox activity (unpublished results). The seed had a total solids content of 364 g/L. A volume of 90 mL was used to inoculate each of the reactors for a final solid concentration of about 65 g/L. Although it was not characterized, the sand contained in the seed was used as support for biofilm formation. The medium used for the culture contained the following (van de Graaf et. al., 1995): 2052 mg/L KHCO$_3$, 174 mg/L K$_2$HPO$_4$, 55 mg/L CaCl$_2$, 102 mg/L MgCl$_2$, 2H$_2$O, 2.0 mL of trace solution 1 and 1.0 mL of trace solution 2. Trace solution 1 contained 10 g/L Na$_2$EDTA.2H$_2$O, 5 g/L FeSO$_4$. Trace solution 2 contained: 15 g/L Na$_2$EDTA.2H$_2$O, 0.43 g/L ZnSO$_4$, 0.24 g/L CoCl$_2$.6H$_2$O, 0.99 g/L MnCl$_2$.4H$_2$O, 0.25 g/L CuSO$_4$.5H$_2$O, 0.19 g/L NiCl$_2$.6H$_2$O, 0.22 g/L NaMoO$_4$.2H$_2$O, and 0.014 g/L H$_3$BO$_4$. Na$_2$SeO$_3$ was not included. The reactors were supplemented with the required substrates, nitrite (NaNO$_2$) and ammonia ((NH$_4$)$_2$SO$_4$) at different concentrations. In order to prevent inhibition of the Anammox activity by nitrite toxicity (van de Graaf et. al., 1995), nitrite was fed in all reactors at a level lower than the reported toxic limit of 5 mM (3.3 mM or 150 mg NO$_2^{-}$/L or 45.6 mg NO$_2^{-}$-N/L). Ammonia was fed at two different levels, two reactors at a low concentration (about 130 mg NH$_4^{+}$/L, or 7.2 mM, or 100 mg NH$_4^{+}$-N/L) and the other two at a high concentration (350 mg NH$_4^{+}$/L, or 19.4 mM, or 272 mg NH$_4^{+}$-N/L). The pH of the mineral solution was about 8.0 and it did not change during the experiment, due to the relatively high concentration of the bicarbonate (KHCO$_3$) buffer. The reactors were amended with trace amounts of intermediates of the Anammox reaction (Figure 2) in the form of hydrazine sulfate ((NH$_2$)$_2$H$_2$SO$_4$) at a concentration of 50 μM and hydroxylamine sulfate ((NH$_3$OH)$_2$SO$_4$) at a concentration of 50 μM. Addition of low levels of these intermediates was reported to initiate Anammox activity (Strous et. al., 1999b).

The activity of the Anammox bacteria was monitored by analyzing the concentrations of ammonia and nitrite at different times. Analyses were performed by colorimetric methods using ready-to-use powder pillows from Hach Company (Loveland, CO).
Figure 3: Schematic of the Anammox reactor set-up

The side arm of each reactor was connected to a plastic tubing to direct the exhaust gases to a 1-L Erlenmeyer filled with water, placed at a lower height than the reactor to prevent backpressure. To ensure anaerobic conditions, contamination with O₂ was avoided by firmly closing each flask with a rubber stopper. The stopper had two holes, one for sampling and the other for gas input. The sampling port consisted of a syringe body tightly inserted through the hole with valve at the outside end. Small-diameter plastic tubing with a needle attached to one end was inserted through the syringe body to reach the contents of the reactor minimizing the dead volume during sampling. The gas input port consisted of a syringe body tightly inserted through the hole. Plastic tubing was passed inside the syringe to reach the bottom of the reactor. A screw clamp was placed at the outside end of the tubing. Anaerobic conditions were established by flushing with N₂ gas for at least 15 min.

Results

Figure 4 shows the concentration profile for nitrite in the four Anammox reactors. The nitrite concentration was replenished back to 150 mg NO₂⁻/L as soon as its depletion was observed. Nitrite was readily consumed in each of the reactors. However, in the case of ammonia (Figure 5), its concentration did not change significantly during the first 30 days of the culture. In fact, it tended to increase between 12 and 24 days. After the 26th day, the ammonia concentration started to slowly decrease, which showed the possibly of Anammox activity. The rate of nitrite consumption was estimated and plotted in Figure 6. This plot revealed that regardless of the level of ammonia, the rate of nitrite consumption reached a maximum of about 1.3 mg NO₂⁻/L.hr after approximately 15 days, and then started to decrease and level off to about 0.6 mg NO₂⁻/L.hr.
These results suggested the possibility that nitrite was consumed by a consortium of microorganisms including Anammox bacteria, denitrifiers, and dissimilatory nitrite reducers. In fact, the initial observation of an increase in the ammonia concentration could be attributed to the activity of the dissimilatory nitrite reducers and ammonification of organic nitrogen present in the original biomass from the seed. Nevertheless, a population shift seemed to begin after 30 days, since the rate of nitrite consumption started to decrease and shortly after the ammonia concentration was being lowered in the culture. Hence, it appears that the population of Anammox bacteria is starting to be sufficiently high to compete with the other microorganisms.

The Anammox reactors will be kept under observation until Anammox bacteria show significant Anammox activity. In this preliminary investigation, the Anammox activity has
been estimated from the consumption of nitrite and ammonia. Rates of consumption of the two substrates can be compared to values reported in the literature.

\[
\begin{align*}
\text{mg NO}_2/L\text{hr} \\
\text{Elapsed time (d)}
\end{align*}
\]

Figure 6: Rate of nitrite consumption in Anammox reactors

**CONCLUSIONS**

The animal waste treatment system proposed has the potential to overcome problems in current waste handling and disposal methods for wastewaters generated at animal feeding operations. This study aims to evaluate the technical feasibility of the proposed system to (1) eliminate the limitations of conventional anaerobic processes, (2) reduce environmental impact of the high residual nitrogen content, (3) provide a source of alternate low-cost energy, (4) significantly reduce odors, and (5) improve overall quality of the liquid waste.

**REFERENCES**


Supercritical Carbon Dioxide Treatment of Lignocellulosic Biomass to Enhance Cellulose Hydrolysis

Muralidhar Reddy Mallem, David J. Dixon, and Patrick C. Gilcrease
Dept. of Chemical and Biological Engineering, South Dakota School of Mines and Technology, Rapid City, SD, USA 57701

Abstract

Lignocellulosic materials are the most abundant renewable organic resources present on earth, containing cellulose, hemicellulose, and lignin as their main constituents. Production of ethanol from these lignocellulosic materials is of major commercial interest, but extensive research is still needed to develop an effective cellulose-hemicellulose conversion process. Softwood (Ponderosa Pine saw dust) was used as the substrate in this research for two reasons: (1) its abundance in western South Dakota as waste forest thinnings and (2) its low cost when compared to other raw materials such as corn or other lignocelluloses. Effective pretreatment of softwood is essential for achieving higher ethanol yields via enzymatic hydrolysis and subsequent fermentation.

The present study involves the pretreatment of Ponderosa Pine with supercritical carbon dioxide (SC CO$_2$) at different conditions (such as temperature, time, pressure and moisture content) to improve glucose and ethanol yields. The effectiveness of SC CO$_2$ was investigated by carrying out enzymatic hydrolysis on the pretreated softwood. The effect of pretreatment temperature, pressure, time, scale-up, and moisture content on glucose yields is discussed. The average glucose yields obtained for our optimized pretreatment conditions ranged from 62.97 ± 2.38 to 91.61 ± 3.64% of total glucose present. In comparison; glucose yields for unpretreated Ponderosa Pine were only 11.75 ± 0.75%. Pretreatment mechanisms for this process, along with implications for process scale-up are discussed.

Introduction

Ethanol, also known as ethyl alcohol or simply alcohol, is a colorless liquid having a faint odour [2]. “The use of ethanol as a transportation fuel has as long a history as the car itself. It began with the use of ethanol in the internal combustion engine invented by Nikolas Otto in 1897 [1].” Ethanol is considered to be a renewable energy source as the raw materials that are used for the production of ethanol like sugar-based feed stocks (sugar cane juice, sugar beet, molasses), starchy products or cereals (corn, wheat, maize, potatoes), and cellulose sources (wood, rice straw, plant material waste) are replenished each year by the sun [2].

The production of ethanol throughout the world received considerable attention during the 1973 oil crisis as OPEC quadrupled the selling price of crude oil. Many countries started research in an effort to develop economical ethanol production from available renewable sources. However, the interest in ethanol production decreased as the selling price of crude oil went down. Following the second oil crisis in 1979, many countries have investigated/developed the production of alternative fuels [1]. Brazil produces about 3.2 billion gallons of ethanol from sugarcane for automotive use every year, and the US agriculture industry provides approximately
1.3 billion gallons of ethanol from corn and other starch-rich grains to produce clean burning high oxygenate fuels. It is predicted that by the year 2010, renewable biofuels could provide 12 billion gallons of ethanol a year, approximately 10% of the total fuel used for automotive transportation in the US [15]. Many Brazilians fuel their cars with pure ethanol, and the rest use a 25% ethanol blend [2]. Ethanol-blended fuels such as E85 (85% ethanol and 15% gasoline) can reduce the net emissions of greenhouse gases like carbon monoxide and hydrocarbons by as much as 37.1% whereas E10 (10% ethanol and 90% gasoline) reduces greenhouse gases by up to 3.9% [2].

Lignocellulosic materials such as wood, agricultural residues, and herbaceous crops contain cellulose, hemicellulose, lignin, and so-called extraneous materials [1, 2]. Extraneous materials are classified as extractives or nonextractives based on their solubilities in water and organic solvents [2]. Both deciduous (hardwood) and coniferous (softwood) tree species are categorized as woody biomass. Ponderosa Pine sawdust, a species of softwood, was used as a substrate in this research. Due to the high lignin content associated with softwoods, it is essential to perform pretreatment prior to enzymatic hydrolysis to achieve reasonable ethanol yields [7]. If tree bark is excluded from the analysis, softwoods show an average composition of 42% cellulose, 21% lignin, 20% hemicellulose, and 6% extractives in pine, spruce, alder, aspen, and birch [1].

Cellulose, a polymer of D-glucose, is the main component of wood [3, 6]. Cellulose is a long, unbranched chain of D-glucose with a molecular weight between 50,000 and 1 million daltons. Glucose monomers are linked together by β-1, 4 glycosidic linkages, which are more resistant to enzymatic hydrolysis compared to the α-1, 4 glycosidic linkages found in starch [4]. A molecule of water is lost when glucose units are linked together into polymer chains, resulting in the chemical formula of cellulose as C₆H₁₀O₅ for each monomer unit of “glucan” [1]. Hemicelluloses belong to a group of heterogeneous polysaccharides having a random, amorphous structure with little strength. Hemicelluloses comprise approximately 15 - 30% of the dry weight of wood. Hemicelluloses can be easily hydrolyzed by dilute acid or base to their monomeric components which include hexoses (galactose and mannose), pentoses (arabinose and xylose) and small amounts of rhamnose, glucuronic acid, methyl glucuronic acid and galacturonic acid [1, 3, 5]. The composition and structure of hemicelluloses in softwoods are different from hardwoods, which affects both the sugar composition of hydrolysates and potential inhibitor formation. Examples of inhibitors are furfural and 5-hydroxymethylfurfural (HMF). These inhibitors are the thermal degradation products of xylose and glucose, respectively. Lignin is a complex molecule composed of polymerized phenylpropanoid acids in a three-dimensional structure [1, 3]. The lignin content of softwoods is appreciably greater (25%-35%) when compared to hardwoods (18%-25%), straws (10%-20%), and some agricultural residues (10%-15%) [3]; as such, the enzymatic digestibility of softwoods is lower when compared to hardwoods or other residues [3].

Pretreatment is an essential and a crucial step to improve overall yields of an ethanol fermentation process. The purpose of pretreatment is to disrupt the structure of the lignin-carbohydrate complex, thereby increasing the accessible surface area and decreasing the crystallinity of cellulose. More accessible surface area and low crystallinity of the cellulose improves overall enzymatic hydrolysis rates and subsequent ethanol fermentation yields.
Pretreatment is considered to be successful when it meets the following requirements [12, 13, 14]:

- Enhances the formation of sugars or the ability to subsequently form sugars by enzymatic hydrolysis.
- Avoids the degradation or loss of carbohydrates.
- Avoid the formation of inhibitory compounds such as furfural and hydroxymethyl furfural, which are inhibitory to the fermentation yeast.

The overall goal of pretreatment research is to find an effective technique for the economical conversion of biomass to ethanol. This research will focus on the supercritical carbon dioxide (SC CO₂) pretreatment of Ponderosa pine in a batch reactor as a function of temperature, pressure, moisture content, and pretreatment time. The effects of scale-up and storage temperature following pretreatment will also be investigated.

Supercritical fluid biomass pretreatment is a technology that uses supercritical fluid as a solvent [8]. Above the critical point, a supercritical fluid (SCF) possesses properties similar to both a gas and a liquid. In the last few decades, supercritical fluids have gained considerable attention for a variety of processes and technologies such as nutraceutical extraction and phospholipid separation [9]. SC CO₂ is one of the most commonly used SCFs because of its inert, inexpensive, non-toxic, non-flammable, readily available and environmentally friendly nature [8, 9, 10, 11]. Its critical temperature (Tₐ = 31.1 °C) and critical pressure (Pₐ = 74 bar or 1073 psi), which can be readily attained with available equipment, allows its use at relatively mild temperatures and moderate pressures [9, 10]. Other fluids having critical parameters close to SC CO₂ such as propane or ammonia are either difficult to handle, difficult to obtain in pure form, toxic, or give rise to highly reactive or explosive mixtures [10].

The effect of SC CO₂ pretreatment on Aspen and Southern Yellow Pine at various pretreatment conditions and different moisture contents was investigated by Kim and Hong [11]. The sugar yields (% theoretical maximum) obtained from the SC CO₂ treatment (at 165 °C, 214 bar for 30 min) of Aspen and Southern Yellow Pine were 84.7 ± 2.6 and 27.3 ± 3.8%, versus 14.5 ± 2.3 and 12.8 ± 2.7% for the untreated samples, respectively. Results were very promising with Aspen, which is a hardwood [11].

Materials and Methods

Substrate
Ponderosa Pine (obtained from Black Hills National Forest, South Dakota) was supplied by Baker Timber Products, Rapid City, SD in sawdust form. The sawdust obtained was then classified between USA Standard Testing Sieve Numbers 20 and 30, which corresponds to a particle size between 0.60 and 0.85 mm. Sieved wood particles were then stored in a closed container at room temperature until pretreatment was performed.

Cellulase Enzyme
The cellulase enzyme preparation (SPEZYME CP) was obtained from Genencor International. This was produced from Trichoderma longibrachratum. The specific activity of this preparation was measured as 10.0 FPU/ml. An FPU is defined by IUPAC as the amount of
enzyme needed to produce 2.0 mg of reducing sugar as glucose from 50 mg of filter paper (4% conversion) in 60 minutes [16].

Supercritical Carbon Dioxide Treatment of Ponderosa Pine
Water was added to 2.0 g of Ponderosa Pine to a desired moisture content; the wetted wood was then transferred into a 18 ml high pressure pretreatment vessel (stainless steel 304). After checking for leaks in a water bath, the pretreatment vessel was attached to a high pressure line inside a Thelco Laboratory oven. The temperature inside the oven was measured using an Omega 869 Thermometer (100 Ω platinum RTD). After the oven reached the desired temperature, the pretreatment vessel was pressurized with CO\textsubscript{2} to the desired pressure and left inside the oven until the pretreatment time was over. Non-refrigerated samples (shown in Figures 1, 2, 3, 4, and 5) were either digested within 2 hours after pretreatment or stored at room temperature for about 7 days before enzymatic hydrolysis was performed. Refrigerated samples shown in Figure 6 were stored at 4 °C until enzymatic hydrolysis was performed.

Enzymatic Hydrolysis of Pretreated Ponderosa Pine
Enzymatic hydrolysis was performed using a standard National Renewable Energy Laboratory (NREL) procedure by transferring 0.233 g of wet, pretreated biomass into a sealable test tube to which 1 ml of citrate buffer, 8.75 ml of distilled water, and 0.25 ml of cellulase enzyme were added [18]. The test tube was sealed with a cap and then placed in an incubator with a heavy duty rotator (speed 5 rpm) at 50 °C for 72 hr. Samples were centrifuged (using a Beckman Coulter Microfuge) at 13000 rpm for 15 min; the supernatant was passed through a 0.2 μm non-cellulosic syringe filter before transferring into HPLC vials. Samples that were not tested immediately were stored at -20 °C to prevent microbial growth.

The total available glucose present in the Ponderosa Pine was determined using a standard National Renewable Energy Laboratory (NREL) protocol [17]. The maximum available glucose obtained from 1 g of dry Ponderosa Pine sample was 0.42 g.

Determination of Glucose
High Performance Liquid Chromatography was used to measure the glucose concentration in each hydrolysate. The HPLC system used in this research consisted of a System Gold® 508 autosampler (Beckman Coulter), a 125 solvent module pump (Beckman Coulter), an intelligent refractive index detector (Jasco RI-1530), and a Model 105 column heater (Timberline Instruments). Data acquisition and analysis was performed using 32 Karat software (Beckman Coulter). A Biorad Aminex® HPX-87H Ion Exclusion column (300 mm x 7.8 mm) was used for the separation of glucose from other components in the sample. Separations were performed at 65 °C with 5 mM H\textsubscript{2}SO\textsubscript{4} mobile phase flowing at 0.6 mL/min.

Statistical Analysis
All the data is presented as the mean ± one standard deviation. Each error bar represents one standard deviation from the mean for four samples (2 hydrolysis tubes per wood sample, with 2 HPLC samples per tube). A t-Test (Two Sample Assuming Equal Variances) was performed at 95% confidence level to compare the means of experimental data groups using Microsoft Excel 2002. Differences with P values of 0.05 or less were considered significant, and are reported as such.
Results and Discussion

This section examines the effect of various pretreatment conditions such as temperature, pressure, moisture content, time, and scale-up on percent of theoretical glucose recovery from enzymatic hydrolysis. The efficiency of SC CO\textsubscript{2} treatment was investigated by performing pretreatment at various conditions throughout the course of this research.

Effect of Pressure

The percent theoretical glucose recoveries following pretreatment and enzymatic hydrolysis are shown as a function of CO\textsubscript{2} pretreatment pressure in Figure 1. From this Figure, it is clear that the increase in average glucose recovery from 68.21 ± 8.30 to 72.21 ± 0.21% was not statistically significant (determined using t-test at 95% confidence interval) when the pressure increased from P\textsubscript{2} to P\textsubscript{2}+87 bar, respectively. The glucose recovery obtained without CO\textsubscript{2} pressure was 66.39 ± 7.53%, which was statistically the same compared with the pressurized runs. There was no significant effect of CO\textsubscript{2} pressure on glucose recoveries. From these results, it appears that carbonic acid was not the main mechanism responsible for achieving high glucose recovery for the 0, P\textsubscript{2}, and P\textsubscript{2}+87 bar runs.

![Figure 1: Effect of CO\textsubscript{2} pressure on biomass pretreatment. Each error bar represents one standard deviation from the mean for four samples (2 hydrolysis tubes per pretreated wood sample, with 2 HPLC samples per tube). Average % theoretical glucose recoveries for samples at 0, P\textsubscript{2}, and P\textsubscript{2}+87 bar were 66.39 ± 7.53, 68.21 ± 8.30, and 72.21 ± 0.21%, respectively.](image)

Effect of Temperature

The percent theoretical glucose recoveries following pretreatment and enzymatic hydrolysis are shown as a function of pretreatment temperature in Figure 2. From this Figure, it is clear that the glucose recovery increased (by approximately 32%) with an increase in temperature from T\textsubscript{1} °C to T\textsubscript{1}+12 °C; however, when the temperature was further increased to T\textsubscript{1}+24 °C, there was no statistical change (confirmed from t-test) in the glucose recovery. The main reason for increased glucose recoveries at higher temperatures could be the process of autohydrolysis. Autohydrolysis of hemicellulose sugars can occur at higher temperatures (above...
160 °C) when acetic acid is formed from the cleavage of acetyl groups within the biomass [3]. Autohydrolysis is also a major mechanism in steam explosion treatment where the temperatures are held at 160 to 260 °C with saturated steam from ten seconds to several minutes [1, 3]. However, sugar degradation products such as furfural (from xylose) or hydroxymethylfurfural (from glucose) can be formed with the combination of acid and heat, reducing the overall sugar recoveries. Since higher temperatures can lead to the thermal degradation of glucose, this may explain why the glucose recovery did not increase from T1+12 °C to T1+24 °C [1, 13]. The final hydroxymethylfurfural concentration increased from 0.011 to 0.028 g/g dry wood as the pretreatment temperature was increased from T1 °C to T1+12 °C.

![Figure 2: Effect of temperature on near-critical CO2 treatment. Each error bar represents one standard deviation from the mean for four samples (2 hydrolysis tubes per pretreated wood sample, with 2 HPLC samples per tube). Average % theoretical glucose recoveries for samples at T1 °C, T1+12 °C, and T1+24 °C were 30.86 ± 2.98, 68.21 ± 8.30, and 61.78 ± 6.02%, respectively.](image)

**Effect of Time**

The percent theoretical glucose recoveries following pretreatment and enzymatic hydrolysis are shown as a function of pretreatment time in Figure 3. As shown in Figure 3, the glucose recovery was lower (32.66 ± 1.83%) at t1 min as the time may not have been sufficient for an effective pretreatment. At t1+50 min the glucose recovery increased to 68.21 ± 8.30%, and at t1+101 min the glucose recovery was 65.64 ± 4.55%. As the t1 min pretreatment time was low, the autohydrolysis mechanism would not have occurred to a maximum extent leading to lower glucose recovery (32.66 ± 1.83%). The glucose recoveries (68.21 ± 8.30 and 65.64 ± 4.55%) obtained at t1+50 and t1+101 min were not statistically different (confirmed from t-test) given the uncertainties associated with these values. The one explanation for not achieving an increased glucose recovery at t1+101 min could be the enhanced conversion of glucose product to hydroxymethylfurfural (0.03 g/g dry wood).
Figure 3: Effect of pretreatment time on near-critical CO$_2$ treatment. Each error bar represents one standard deviation from the mean for four samples (2 hydrolysis tubes per pretreated wood sample, with 2 HPLC samples per tube). Average % theoretical glucose recoveries for samples at $t_1$ min, $t_1+50$ min, and $t_1+101$ min were 32.66 ± 1.83, 68.21 ± 8.30, and 65.64 ± 4.55%, respectively.

Effect of Moisture Content

The percent theoretical glucose recoveries following pretreatment and enzymatic hydrolysis are shown as a function of moisture content (before pretreatment) in Figure 4. The different moisture contents investigated were $M_1$, $M_1+10$, $M_1+15$, and $M_1+23$ wt%. From this figure, it is clear that the percent theoretical glucose recovery obtained for the $M_1$, $M_1+10$, and $M_1+15$ wt% moisture content samples was approximately 20% greater compared with the $M_1+23$ wt% moisture content sample. The glucose recovery trend observed for this experiment was the same as reported by Kim and Hong [11], where glucose recoveries obtained with Southern Yellow Pine at 165 °C, 214 bar (3100 psi) and 60 min for 57 and 73 wt% moisture biomass were 38% and 22%, respectively. These results indicate that the moisture content plays a vital role in this pretreatment, presumably due to its effect on acid concentration during autohydrolysis. The lower glucose recoveries (57.17 ± 1.28, 40.71 ± 1.79%) at $M_1+15$ and $M_1+23$ wt% moisture levels may be due to the dilution of acetic acid necessary for the hydrolysis of carbohydrates. Kim and Hong [11] conducted SC CO$_2$ treatment on dry Aspen and found that the percent theoretical sugar recovery was the same as for untreated Aspen. Therefore, no pretreatment was performed with dry biomass at these conditions in this research study. Since there was only one pretreatment experiment performed at each of the $M_1+10$, $M_1+15$, and $M_1+23$ wt% moisture levels, future experiments should be performed on the biomass at these moisture contents to further verify the glucose recovery trend shown in Figure 4.
Figure 4: Effect of moisture content on SC CO₂ treatment. Each error bar represents one standard deviation from the mean for four samples (2 hydrolysis tubes per pretreated wood sample, with 2 HPLC samples per tube). Average % theoretical glucose recoveries for samples at M₁, M₁+10, M₁+15, and M₁+23 wt% moisture content were 62.97 ± 2.38, 64.74 ± 1.18, 57.17 ± 1.28, and 40.71 ± 1.79%.

Effect of Scale-up

This experiment was performed by increasing the amount of biomass in each pretreatment batch. The different biomass weights investigated for pretreatment were 2.00, 4.66, and 6.00 g of dry wood. Reactor tubes of 50 ml total volume (Autoclave Engineering 25.4 mm OD x 17.5 mm ID x 203 mm long pipe, with sealing caps) were used for the pretreatment of 4.66 and 6.00 g batches of wood. The percent theoretical glucose recoveries following pretreatment and enzymatic hydrolysis are shown as a function of biomass weight pretreated in Figure 5. It is clear from this Figure that the average percent theoretical glucose recovery decreased from 62.97 ± 2.38 to 15.41 ± 3.25% with an increase in the weight of biomass from 2.00 to 6.00 g treated. One possible explanation is that the temperature inside the larger high pressure autoclave vessel might not have equaled the outside oven temperature during the pretreatment as the heat transfer resistance was larger when compared with the 18 ml pretreatment cell. Due to this increased resistance, the wood present inside the center of the vessel may not have reached the desired pretreatment temperature. To justify the above hypothesis, an experiment was performed to measure the temperature of the wood mass inside an even larger (~50.80 mm diameter) static Parr reactor. Significant heat transfer limitations were observed, as the temperature of the wood inside the reactor vessel did not reach the desired temperature even after 220 min. There was also a continuous increase in pressure during the pretreatment indicating that the temperature of the sample was increasing during the run.
Figure 5: Effect of process scale-up on SC CO₂ treatment. Each error bar represents one standard deviation from the mean for four samples (2 hydrolysis tubes per pretreated wood sample, with 2 HPLC samples per tube). Average % theoretical glucose recoveries for all samples which used 2.00, 4.66, and 6.00 g of wood were 62.97 ± 2.38, 46.24 ± 4.66, and 15.40 ± 3.25%, respectively.

**Effect of Refrigeration**

The percent theoretical glucose recoveries following pretreatment and enzymatic hydrolysis are shown as a function of storage conditions following pretreatment in Figure 6. Samples labeled "refrigerated" in Figure 6 were stored in the refrigerator at 4 °C for a period of 13 days before enzymatic hydrolysis was performed. From this figure, it is clear that the average percent of theoretical glucose recovery for all refrigerated samples (91.61 ± 3.64%) was greater than that of non-refrigerated samples (68.18 ± 8.37%). From these results, we can speculate that there might be additional biomass degradation taking place during refrigerated storage, increasing the surface area of cellulose for enzyme attack. If autohydrolysis has occurred, it would provide additional time for wood with acid during the 13 day storage period at 4 °C. Autohydrolysis would not have occurred to a maximum extent with the non-refrigerated wood (digested after 7 days) as the moisture content in the wood might have partially evaporated (due to the storage of wood at room temperature).
Conclusions

This research study indicates that the SC CO₂ pretreatment of biomass (Ponderosa Pine saw dust) was promising, as glucose recoveries following enzymatic digestion ranged from 62.97 ± 2.38 to 91.61 ± 3.64% of total glucose present. Autohydrolysis of hemicellulose sugars appears to be the main mechanism for achieving higher glucose recoveries; CO₂ pretreatment pressure had no significant effect on final glucose recoveries. Glucose recovery increased from 30.86 ± 2.98 to 68.21 ± 8.30% when the pretreatment temperature was increased from T₁ to T₁ + 12 °C. Lower autohydrolysis rates resulted in lower glucose recoveries at temperature T₁. When the temperature was increased from T₁ + 12 °C to T₁ + 24 °C, there was no change in glucose recovery (61.78 ± 6.02%); apparently, increases in the autohydrolysis rates were offset by increased thermal degradation of the glucose product to hydroxymethylfurfural at T₁ + 24 °C. The glucose recovery increased from 32.66 ± 1.83 to 68.21 ± 8.30% when the pretreatment time was increased from t₁ to t₁ + 50 min. But when the pretreatment time was further increased to t₁ + 101 min, there was no change in the glucose recovery (65.64 ± 4.55%). Again, increased thermal degradation of glucose to hydroxymethylfurfural (0.03 g/g dry wood) may have offset any increase in hemicellulose/cellulose hydrolysis.

The glucose recovery obtained at M₁ wt% moisture content (62.97 ± 2.38%) was statistically the same compared with the glucose recovery (64.74 ± 1.18%) obtained at M₁ + 10 wt% moisture content. Moisture content plays a vital role in both the autohydrolysis and carbonic acid hydrolysis of carbohydrates. Lower glucose recoveries (57.17 ± 1.28, 40.71 ± 1.79%) were obtained at M₁ + 15 and M₁ + 23 wt% moisture contents, perhaps due to the dilution of acetic acid, which adversely affected the acid hydrolysis of carbohydrates.
Kim and Hong [11] dried the Southern Yellow Pine sample using a 45 °C vacuum oven before SC CO₂ pretreatment was performed on it. NREL argues that drying permanently collapses the pore structure of wood making it difficult for digestion. In this study, pretreatment was performed on the wood (by adjusting the moisture content to 57 wt%) that was directly obtained from the saw mill without drying it. This could be a possible explanation for achieving higher glucose recoveries in this research compared to Kim and Hong.

The optimum pretreatment conditions not only increased the glucose recoveries but also led to the formation of hydroxymethylfurfural, which inhibits the fermentation yeast. Therefore, it is important to optimize the pretreatment conditions such that hydroxymethylfurfural and/or furfural formation is minimized.

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Electrochemical Behavior of Ferrocene-modified Poly(ethylenimine) Redox Polymers

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Abstract

The preparation and characterization of novel ferrocene redox polymers based on the coupling of ferrocenecarboxyaldehyde to both linear and branched poly(ethylenimine) are described. The electrochemical behavior of both the ferrocene-modified linear poly(ethylenimine) (Fe-LPEI) and the ferrocene-modified branched poly(ethylenimine) (Fc-BPEI) was investigated by cyclic voltammetry (CV). At low pH (< 5) the linear polymer (Fe-LPEI) exhibited multiple redox wave behavior (~150 & 340 mV vs SCE) in solution which transitioned into a single redox wave (~340 mV) at pH > 7. In contrast the branched polymer (Fc-BPEI) exhibited a single redox wave (~350 mV) at both low and high pH. When the linear and branched polymers were crosslinked with ethylene glycol diglycidyl ether (EDGE), they exhibited multiple redox wave behavior (~300 mV and ~550 mV) at pH > 7. As the crosslink density increased the magnitude of the redox wave at 560 mV increased and the films became increasingly unstable.

INTRODUCTION

The development of new and novel redox polymers has been the focus of considerable research over the last 20 years due to their potential use in electrocatalysis\textsuperscript{1}, photoelectrochemistry\textsuperscript{2}, biosensing applications\textsuperscript{3-6}, drug delivery\textsuperscript{7,8} batteries\textsuperscript{9}, and biofuel cells\textsuperscript{10,11}. Various polymer backbones and redox couples have been utilized in the development of these novel redox polymers. Among the more popular polymer backbones utilized are poly(vinylpyridine)\textsuperscript{1,3}, poly(vinylimidazole)\textsuperscript{12,13}, and poly(allylamine)\textsuperscript{14,15}, while ferrocene, osmium, and ruthenium are the most popular redox mediators. Previous studies have suggested that the following characteristics are among the most desired for redox polymers: (i) polymer flexibility or high segmental mobility\textsuperscript{16,17}, (ii) a high degree of functional density on the polymer to facilitate modification and crosslinking\textsuperscript{18}, and (iii) a polymer that hydrates well\textsuperscript{19}.

Poly(ethylenimine) (PEI) is an attractive candidate to serve as a redox polymer backbone for all of the reasons listed above. PEI can be obtained in either a branched (BPEI) or a linear (LPEI) form (Figure 1). Both the linear and branched forms have glass transition temperatures (LPEI Tg = -35°C, BPEI Tg = -50°C) that are below that of currently utilized redox polymer backbones such as poly(vinylpyridine) (Tg = +142°C)\textsuperscript{20} or poly(vinylimidazole) (Tg = +163°C)\textsuperscript{21}. This suggests that at room temperature or higher they should exhibit a high degree of segmental mobility and thus have enhanced electron transport. A second attractive feature of PEI polymers
is that they have a high density of amines in their structure that allows one to easily modify the polymer. As shown in Figure 1, one can easily attach different redox moieties to the polymer (e.g. Ferrocene, Ruthenium, or Osmium complexes) through the primary (BPEI) and secondary (LPEI) amine groups. This should also allow one to vary the degree of redox center substitution and control crosslink density.

Recently there have been a few reports on the development of new redox polymers based on poly(ethylenimine) backbones. Huan et al., coupled Toluidine Blue O moieties to the primary amines of BPEI through a terephthaloyl coupling in the development of sensors for the electrocatalytic oxidation of NADH. Alternatively, Lui coupled ferrocene carboxaldehyde to BPEI and incorporated these redox polymers into polyelectrolyte multilayer films via a layer-by-layer deposition technique. Chuang et al., also coupled ferrocene carboxaldehyde to BPEI but incorporated these redox polymers into carbon paste electrodes for glucose sensing. Although the study by Chuang reported the effect of pH on the electrocatalytic response of their sensors to glucose, they did not systematically investigate the effect of pH or crosslinking on the electrochemical behavior of the redox polymer alone. Understanding the influence of pH on PEI-based redox polymers is essential since it is well known that both uncrosslinked and crosslinked forms of PEI undergo a volume collapse at high pH due to hydrogen bonding between PEI chain segments and surrounding water molecules which results in the formation of crystalline hydrates. In contrast, protonated forms of PEI at low pH are in an extended state due to the electrostatically repulsive positive charges on the polymer backbone, and have been shown to be quite soluble in water. In this study we report the synthesis of a novel ferrocene redox polymer based on LPEI and systematically investigate the effects of pH and crosslinking on the electrochemical behavior of both BPEI and LPEI modified with ferrocene. Understanding the redox properties of these polymers and the factors that affect charge transfer through them are critical to maximize the usefulness of these polymers.

EXPERIMENTAL SECTION

Chemicals and Solutions
Ferrocenecarboxaldehyde, poly(2-ethyl-2-oxazoline), and sodium borohydride were purchased from Aldrich. All chemicals and solvents were reagent grade and used as received. Water was obtained from a Nanopure® water purification system and had a specific resistance of 18 MΩ cm. Phosphate buffer solution (PBS, pH 7.4) was prepared by dissolving 8.0 g of NaCl, 1.0 g of Na₂HPO₄, 0.2 g of KCl, and 0.2 g KH₂PO₄ in 1000 ml of water.

Instrumentation
Electrochemical measurements were performed with a bipotentiostat (Model 832) and 3mm glassy carbon electrodes purchased from CH Instruments (Austin, TX). Unless otherwise noted experiments were conducted in a three-electrode cell configuration with a saturated calomel reference electrode (SCE), and a platinum wire counter electrode. Prior to use, all electrodes were polished successively on three grades of alumina (5, 1, 0.3 µm) and washed thoroughly with Nanopure water after each polishing step. Constant temperature (25±1°C) was maintained during the experiments by using a water-jacketed electrochemical cell connected to a circulating water bath. An Accumet® AR25 pH meter (Fisher Scientific) was used to determine the pH of
the larger volume electrochemical solutions, whereas pH test strips were used to indicate the pH of the lower volume polymer solutions used to prepare the redox hydrogels.

Synthesis and Characterization of Fc-LPEI and Fc-BPEI
Linear poly(ethylenimine) (LPEI) (avg. MW ca. 86,000) was obtained by acidic hydrolysis of poly(2-ethyl-2-oxazoline) (avg. MW 200,000), followed by neutralization with sodium hydroxide29. In a round-bottom flask, 0.252 g of LPEI was dissolved in 10 mL methanol and a solution of 0.187 g (0.87 mmol) ferrocenecarboxaldehyde dissolved in 3 mL methanol was added to it dropwise under constant agitation. The resulting dark red solution was stirred for 2 h and cooled in an ice bath. Sodium borohydride (0.033 g, 0.87 mmol) was added, upon which the solution lightened in color. After 1 h, the methanol was removed under reduced pressure and the residue was extracted overnight with diethyl ether to remove any non-reacted aldehyde and ferrocenylmethanol. The ether was then decanted and the residue was washed with diethyl ether before being dried under reduced pressure. The residue was extracted with benzene and insoluble salts were removed by filtration. Finally the solvent was removed from the filtrate under reduced pressure to give ca. 0.170 g (40%) LPEI-Fc. The Fc-BPEI was prepared by the same method as the Fc-LPEI23.1 H-NMR (300 MHz, d₆-benzene): δ 3.9-4.3 (br, Fe ring H), 3.7-3.3 (br, FcCH₂, NH), 2.5-2.9 (br, -CH₂N-) The degree of substitution of the amine hydrogens by ferrocenylmethylene moieties was estimated from the integration ratio of the ferrocenyl proton to polymer methylene backbone signals in the ¹H-NMR spectra of each resulting polymer and found to be ca. 22% for the Fc-LPEI, and ca. 20% for the Fc-BPEI. Differential scanning calorimetry showed the polymer to have a glass transition temperature of ca. -14 °C.

Solution Electrochemistry
Since the Fc-LPEI and Fc-BPEI were not soluble in neutral aqueous solution at room temperature, 20 mg of each polymer was initially dissolved in 10 ml of 0.1 M HCl. The pH of each solution was then adjusted to the desired value using concentrated NaOH. Cyclic voltammetry experiments were performed on the polymer solutions at pH values of 1, 3, 5, 7, 9, and 11, using a Ag/AgCl electrode as the reference at a scan rate of 50 mV/s.

Redox Hydrogel Film Preparation/Electrochemistry
The Fc-LPEI and Fc-BPEI were each dissolved in aqueous solution at pH 3, and the pH was subsequently neutralized with a NaOH solution until the final concentration of the polymer solution was 10 mg/ml. The polymer solutions were mixed with ethylene glycol diglycidyl ether (EGDGE) such that the ratio of nitrogen atoms in the polymer to the epoxide groups on the crosslinker (2 per molecule) was 5:1, 5:2, and 5:4, corresponding to theoretical degrees of crosslinking of 20%, 40% and 80% respectively. Since the actual degree of crosslinking was undetermined, the films will be distinguished by the weight percent of EGDGE in each film (29, 44, and 62 wt% respectively). The electrode surfaces were coated with 3 μl aliquots of each polymer/EGDGE mixture and allowed to dry for 12-14 hours in ambient conditions before electrochemically testing in aqueous solutions of varying pH. The pH of the electrochemical media was adjusted using concentrated HCl and NaOH, and a new electrode film was tested at each pH. Cyclic voltammetry experiments were performed on the polymer films at pH values of 1, 3, 5, 7, 9, and 11, using a saturated calomel electrode as the reference and a scan rate of 50 mV/s.
**Gel Preparation and Swelling**

Highly concentrated polymer solutions (165 mg/ml) were prepared to create gels suitable for handling during swelling experiments. In this case 100-150 mg of polymer was placed in a vial and 0.1 M HCl was added to half the volume required to achieve 165 mg/ml. The vial was placed in a hot water bath and heated until the polymer dissolved. The vial was removed from the heat and HCl was added in small increments. When the solution cooled and the polymer recrystallized, the solution was reheated until dissolution and more HCl was added. This process was repeated until the polymer remained dissolved at room temperature. The remaining volume required to achieve 165 mg/ml was added using 0.2 M HCl to lower the pH of the final polymer solution between 5 and 6.

The concentrated polymer solutions were mixed with EGDGE such that the ratio of nitrogen atoms on the polymer to the epoxide groups on the crosslinker was 5:2 corresponding to a 40% theoretical degree of crosslinking. The resulting polymer/crosslinker mixture was placed in a 1ml syringe whose tuberculin slip tip had been cut off with a razor. Special care was taken to ensure that bubbles were not created when transferring the polymer/crosslinker mixture to the modified syringe. After curing for 48 hours the gels were carefully pushed out of the syringe using the plunger and were allowed to dry in ambient conditions for another 24 hours. The diameter of the gel cylinder was measure at approximately 3 mm using digital calipers and discs measuring approximately 1 mm in length were cut from the cylinder using a razor.

**RESULTS and DISCUSSION**

**Solution Electrochemistry of Fc-LPEI**

Figure 2 shows the cyclic voltammetric behavior of the 22% substituted Fc-LPEI polymer in aqueous solution as a function of pH. At pH 1 the polymer exhibited a well define redox couple at ~150 mv and a less-defined couple at 340 mv. As the pH was increased to 5, the two oxidation peaks appeared to overlap, which resulted in a broad oxidation peak that spanned from 150 to 340 mV. When the pH was increased to 7 the oxidation peak narrowed and a single oxidation peak was observed at 265 mV. Increasing the pH further to pH 9 and 11 resulted in a narrowing of the oxidation peak and the oxidation peak potential shifting to 330 mV and 360 mV, respectively. To verify that the observed effects were due to pH changes and not changes in ionic strength, we doubled the ionic strength (0.2 M) with NaCl and repeated the experiments. There was no change in the electrochemical behavior when the ionic strength was doubled (data not shown). When control CV experiments were performed with non-substituted LPEI, redox activity was not observed in the same potential range, which eliminated the possibility that the observed behavior involved oxidation and reduction of the nitrogen atoms on the polymer backbone.

While the majority of nitrogen atoms in the LPEI backbone are protonated at pH 1, it has been shown that the degree of protonation of LPEI does not reach 100%, even at very low pH\textsuperscript{25-27,30}. Based on this, we hypothesize that the oxidation peak at 170 mV is due to the oxidation of ferrocene molecules attached to, or in close proximity to positively charged nitrogen atoms, while the oxidation peak at 340 mV corresponds to ferrocenes attached to, or in close proximity to neutral nitrogen atoms. As the pH of the solution is increased, the nitrogen atoms on the
polymer backbone are gradually deprotonated and thus the two populations of ferrocene at low pH transition to a single environment at high pH. In addition, the deprotonation results in a shifting of the oxidation peak to higher potentials. Additional support for this hypothesis was provided by using an 8% substituted Fc-LPEI. Figure 3 compares the CV response of the 8% vs 22% substituted polymers. At pH 1 the ratio of the oxidation current at 340 mv to the oxidation current at 170 mv is larger for the 8% substituted polymer (ratio =1.1) than the 22% substituted polymer (ratio =0.8). Hence for the same degree of protonation on the polymer backbone, the polymer with fewer ferrocene sites would be less likely to have ferrocene molecules attached or in proximity to the positively charged nitrogen atoms and their associated counter ions. This produces a relatively lower oxidation current at 170 mV and a relatively higher oxidation current at 340 mV.

We acknowledge that the lower oxidation potential of the ferrocene molecules in regions of high degrees of protonation seems counterintuitive since the resulting ferricinium ion is also positive. Indeed, Alvarez et al showed that in dendrimers with multiple ferrocene centers, the protonation of the nitrogen bridge results in an increase in oxidation potential of the ferrocene molecules\textsuperscript{31}. Nonetheless, similar unusual behavior in ferrocene systems has been reported. These studies have shown that ferrocene molecules are “easier” to oxidize when counter ions are bound to them, since anions bind more strongly to the cationic ferricinium ion\textsuperscript{32,33}. Thus the binding of counter ions to ferrocene molecules results in a negative shift in the redox potential of the Fe/Fe\textsuperscript{+} couple. Based on this mechanism, a possible explanation for the behavior observed in our studies is that at low pH the counter ions associated with the protonated nitrogen atoms make the formation of ferricinium energetically more favorable, resulting in a lower oxidation potential. However at high pH a majority of the ferrocenes will be near a neutral nitrogen, and hence there is no counter ion nearby resulting in a higher oxidation potential.

An additional factor to be considered when interpreting our results is the role that conformational changes in the polymer may be playing as the pH changes. At low pH the nitrogen atoms on the polymer are protonated causing the polymer to be in an extended state. However, as deprotonation occurs, the number of electrostatically repulsive positive charges decreases and attractive hydrogen bonds form between the nitrogen atoms on the polymer backbone and water molecules causing the polymer to collapse. Eventually crystalline hydrates form and the polymer drops out of solution\textsuperscript{24,34}. This phenomenon was observed above pH 11 for the Fc-LPEI. The fact that there was little change in the CV response between pH 7 and 11, suggests that the polymer conformation did not affect its solution electrochemical response significantly.

**Solution Electrochemistry of Fc-BPEI**

A comparison of the electrochemical behavior of the Fc-LPEI in aqueous solution to that of the Fc-BPEI yields a marked difference between the two polymers. Figure 4 shows that at pH 1 the Fc-BPEI exhibits a single oxidation peak at ~400 mv, in contrast to the two oxidation peaks exhibited by Fc-LPEI. In addition the shape of the redox wave and the oxidation peak potential of the Fc-BPEI did not change significantly as the pH was increased to 5 (data not shown). At pH 7 the oxidation peak shifted to ~ 440 mv, and a relatively small shoulder peak appeared around 590 mv. Electrochemical data was only collected up to pH 7 for the Fc-BPEI in solution since this polymer began to precipitate at pH 9. Nonetheless, this data provides valuable
information for the comparison of the electrochemical behavior of the linear and branched ferrocene substituted PEI.

We believe that the structural differences between the two polymers give rise to the discrepancy in their electrochemical behavior in solution. In the branched PEI most of the ferrocene molecules are likely bound to the primary amines, located on the outer edges of the polymer structure. Thus protonation of the nitrogen atoms on the polymer backbone should not have as great an effect on the local ionic environment of each ferrocene. In contrast, the ferrocene molecules on the linear PEI are directly attached to the secondary amines on the polymer backbone, and thus, their local ionic environment is more affected by changes in pH. The effects of these structural differences are observed at pH 1 where the branched polymer exhibits one oxidation peak while the linear polymer exhibits two. Additionally, the oxidation potential of the ferrocene molecules on the branched polymer remains relatively constant as deprotonation occurs while there is a shift to more positive potentials in the linear polymer.

**Electrochemistry of Crosslinked Fc-LPEI Films**

To further explore the possibility that conformational changes in the polymer may be the cause of the observed behavior, we investigated the electrochemistry of a crosslinked film of the linear polymer (Fc-LPEI). Crosslinking of the polymer film should reduce the extent to which the polymer can change conformations as the pH is adjusted. To crosslink the polymer we used either dibromohexane or ethylene glycol diglycidyl ether (EGDGE). Since similar results were obtained with both crosslinkers, only data for films crosslinked with EGDGE are presented. Due to the dynamics of the initial electrochemical testing of redox hydrogels in aqueous solution, such as (i) hydration and swelling of the gel, (ii) leeching of gel components, and (iii) flux of ions, the first 4 scans of each cyclic voltammetry experiment have been omitted; thus, the data in Figure 5 are potential scans 5-10 at each pH.

At pH 1 a small oxidation peak is located around 240 mv followed by a large oxidation peak at 420 mv (Figure 5A). The oxidation peak at 240 mv was only observed at pH 1. In contrast the second oxidative peak progressively shifted to lower redox potentials as the pH was increased to 3 (E_{ox} = 375 mv) and then to 5 (Figure 5B, E_{ox} = 340 mV). In addition, a small oxidation shoulder appeared at ~550 mv at pH 5. It is important to note that the electrode's current response at pH 1 and pH 5 were quite stable, as it did not change significantly with each successive potential scan. At pH 7 (Figure 5C) the second oxidation peak was shifted further to ~325 mv, and a third oxidation peak was now evident at 560 mv. Additionally, the electrode's response at pH 7 was unstable and decreased with each subsequent potential scan. At pH 11 (Figure 5D) the location of the oxidation peaks was unchanged, however, the relative magnitude of the third oxidation peak, and the extent of the instability were much larger than those observed at pH 7.

We hypothesize that the oxidation peak at ~240 mV and a pH of 1, is most likely due to ferrocene molecules located in highly protonated environments where the presence of counter ions results in the stabilization of the oxidized ferrocenes, and hence, a lower oxidation potential, similar to the oxidation of Fc-LPEI in aqueous solution at the same pH. The gel should be in a swollen state at low pH due to the electrostatically repulsive charges on the backbone. The fact that the peak at 240 mV is only observed at pH 1 suggests that under highly acidic conditions a
A "highly" protonated redox environment is created. Although the number of ferrocenes exposed to this environment is small, their redox potential (~240 mV) is distinct from the redox potential of the majority of ferrocene molecules (~375 mV) in the hydrogel. At pH 3 the gel should be still swollen, but slightly less protonated than at pH 1. The lone oxidation peak at 375 mV suggests that (i) any differences in redox environments that may exist at this pH are not electronically distinguishable, or (ii) the global redox environment of the gel is at an intermediate degree of protonation between the two environments at pH 1.

At this time the cause of the third redox oxidation peak that appears at ~560 mV for pH’s > 5 is unknown and further experiments to elucidate the nature of this effect are ongoing. One possible explanation for this peak is that there is electronic communication between neighboring ferrocenes. It is well documented that when ferrocenes are contained in the main chain of redox polymers and closely spaced, substantial interaction between ferrocene centers occurs. The initial oxidation of one ferrocene site makes the subsequent oxidation of a neighboring ferrocene site energetically less favorable and thus requiring a higher oxidation potential. Similarly it has been reported in a Tris(ferrocene)-tren ligand that electrostatic interactions may occur between the electrogenerated positive charge of a ferricinium group and the lone pair of a nitrogen atom connected to a neighboring ferrocene group making the neighboring ferrocene more difficult to oxidize. Furthermore, it is well known that both linear and branched PEI collapse at high pH’s due to hydrogen bonding between water and the nitrogen atoms in the polymer. Thus as the polymer collapses, the distance between ferrocene groups decreases and the oxidation of one ferrocene molecule results in an increase in oxidation potential of an adjacent ferrocene molecule. The fact that the oxidation peak at ~360 mV decreases with time, while the peak at ~560 mV increases suggests that there is a transition from one ferrocene environment to a second ferrocene environment. Additionally, the crosslinking of the polymer may result in the formation of a “tighter” complex into which the influx of counter ions upon the formation of ferricinium ions is more difficult. Since electroneutrality must be maintained in the film, this third peak may be the result of poor movement of counter ions.

Effect of Crosslinker Concentration on Fc-LPEI Films
Since crosslinking of the film resulted in a significant change in the electrochemical behavior of the linear polymer we investigated the effect of crosslinker concentration. Figure 6 shows the electrochemical behavior of Fc-LPEI gels crosslinked with varying amounts of EGDGE in aqueous media at pH 7. Increasing the amount of crosslinking in these gels increases the relative magnitude of the oxidation peak at 560 mV. Furthermore, the onset of the oxidation peak at 560 mV occurs at a progressively lower pH with an increase in crosslinker concentration (not shown). While the gel with 29 wt% EGDGE shows a slight peak in this potential region at pH 5, the gel with 62 wt% EGDGE shows a very prominent peak with a relatively large oxidation current at 560 mV. This suggests that this third oxidation peak is highly dependent on the crosslink density of the film. As the crosslinker concentration increases both the rigidity of the film will increase and the distance between ferrocene sites will be reduced. In addition a tighter crosslinked film may hinder the influx and motion of counter ions in the film.

Crosslinked Fc-BPEI Film Electrochemistry
We next investigated whether crosslinking of the branched PEI polymer would cause similar changes in its electrochemical behavior. Figure 7 shows the electrochemical behavior of
crosslinked Fc-BPEI films as a function of pH. The Fc-BPEI crosslinked with EGDGE behaved quite similarly to the crosslinked linear polymer in the following ways: (i) the current response was stable at low pH, (ii) as the pH is increased the emergence of the oxidation peak at ~560 mv appeared, and (iii) the current response became unstable at pH > 7. However there were some notable differences between crosslinked films of the branched and linear polymers: (i) for the branched polymer there was no oxidation peak at ~170 mv at pH 1, (ii) the relative magnitude of the oxidation peak at 560 mv was lower in the branched polymer, and (iii) the branched polymer produced a higher current response.

We hypothesize that these differences were, again, related to the structural dissimilarities in the linear and branched polymers, specifically the ferrocene molecules likely being attached to the primary amines, located on the outer edges of the branched polymer. These ferrocene molecules may be less susceptible to highly protonated environments at low pH, and being bound to or within hydrogen bond lattices at higher pH. The increased current could be due to the electrochemical accessibility, or the increased segmental mobility of the ferrocene molecules, or some combination of these two phenomena. The effect of crosslinker concentration was also studied for the branched polymer and was found to have a similar effect as illustrated with the linear polymer.

CONCLUSIONS

In this study we report the synthesis of a novel redox polymer based on the modification of LPEI with ferrocene moieties. We systematically investigated the effect of pH and crosslinking on both Fc-LPEI and Fc-BPEI. Using cyclic voltammetry we were able to demonstrate that pH has a major influence on the electrochemistry of Fc-LPEI whether in solution or in a crosslinked film. In contrast the electrochemistry of Fc-BPEI in solution appeared to be sensitive to pH only at pH’s 7 and higher. We also determined that the degree of crosslinking influenced the electrochemical behavior of both Fc-LPEI and Fc-BPEI. As the crosslink density increased the electrochemical response of the films became increasingly unstable. At this time the exact cause of this instability is unknown, but may be related to the morphological changes that occur as the polymer transitions to a collapsed state. Nonetheless, these materials appear to hold great promise for applications such as biosensors. Upon the modification of the polymers with the ferrocene redox moiety, both the linear and branched forms of PEI maintain segmental mobility, and become electrochemically active. Additionally, they are both readily crosslinked and the crosslink density is easily controlled. In lieu of these characteristics, biosensor development based on these redox polymers, similar to the work performed by Chuang et al, is underway, and preliminary results show these materials to be of value.

REFERENCES


Figure 1: Linear and Branched Poly(ethylenimine) Modified with Ferrocene

LPEI-Fc (Linear)  
BPEI-Fc (Branched)
Figure 2: Solution electrochemistry of Fc-LPEI at 50 mV/sec (A) pH 1, (B) pH 5 and pH 7, (C) pH 9 and pH 11
Figure 3: Solution electrochemistry of 8% and 22% substituted Fe-LPEI at pH 1 and 50 mv/sec

Figure 4: Solution electrochemistry of Fe-BPEI at pH 1 and pH 7, 50 mv/sec
Figure 5: Electrochemistry of Fe-LPEI film crosslinked with EGDGE (29 wt%) at 50 mV/sec. Data shown are potential scans 5-10. (A) pH 1, (B) pH 5, (C) pH 7, and (D) pH 11
Figure 6: Electrochemistry of Fe-LPEI film crosslinked with varying amounts of EGDGE at 50 mV/sec and pH 7: (A) 29 wt% EGDGE, (B) 44 wt% EGDGE, (C) 62 wt% EGDGE.
Figure 7: Electrochemistry of Fe-BPEI film crosslinked with EGDGE (29 wt%) at 50 mV/sec. Data shown are potential scans 5-10, (A) pH 1, (B) pH 5, (C) pH 7, and (D) pH 11.
Recovery and purification of recombinant protein from transgenic corn by aqueous two-phase partitioning

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Abstract
Corn has been used as an expression host for several recombinant proteins with potential for large-scale production. Cost-effective downstream initial recovery, separation and concentration remain a challenge. Aqueous two-phase (ATP) partitioning has been used to recover and concentrate proteins from fermentation broths and offers advantages for integration of those steps with biomass removal.

An integrated procedure, which combines extraction and partitioning into one step, was compared to partitioning of separately prepared clarified extracts (two-step procedures). The results of the integrated procedure showed less protein extracted and lower protein partitioning to the upper phase than in the two-step procedures. A model protein, lysozyme, was added to the integrated extract to assess separation selectivity of this method for a protein that had previously been enriched by the two-step procedures. Phase ratio and NaCl addition were manipulated to optimize separation by ATP partitioning. NaCl addition and lowering the phase ratio enhanced the separation of lysozyme from corn proteins.

Keywords: partitioning; aqueous two-phase; corn; protein recovery; extraction

1. Introduction
Utilization of transgenic crops as expression hosts offers several distinct advantages over more widely-used hosts: ability to carry out post-translational modifications to produce active proteins, easier scale-up, and established production methods [1-3]. Furthermore, the ability to target recombinant proteins to specific storage organs or tissues (e.g. seeds and tubers) allows for prolonged stable storage and may reduce the number of contaminants in extracts [1, 4-6].

Corn is preferred over other plant hosts for its lower water-soluble protein content. Furthermore, specific target tissue expression will benefit the downstream purification process via the potential of initial milling and dry fractionation to reduce contaminants and enrich the target protein concentration. Proper choice of the targeted tissue will increase these benefits. For instance, if a protein is targeted to endosperm, protein extraction with a neutral salt buffer will leave nearly 80% of the native proteins in the residue. For germ, only 30% of the native proteins would remain in the residue [6]. Furthermore, an endosperm extract would have much less oil than a germ-rich or whole kernel extract, which would also simplify purification [6]. Using corn fractionation by dry milling to improve purification performance has been studied for transgenic aprotinin and GUS production. Targeting expression to germ combined with dry fractionation of the grain to produce a germ-rich fraction resulted in a starting material with ten-fold higher GUS/solids ratio in the germ fraction than in the whole corn [1].

Protein recovery from the starting material generally includes extraction, clarification, protein capture, purification, and polishing. The overall production cost is mainly determined by the efficiency of the initial capture and purification steps where feed volumes are large until
biomass solids and oils are removed and the protein is concentrated [1, 6]. Therefore, more efficient initial concentration and separation procedures need to be developed for recombinant protein recovery from transgenic corn.

For non-plant hosts, ATP partitioning has offered an alternative for large-scale protein extraction and recovery [7-9]. The advantage of this method is the potential for integrating extraction, clarification, and separation in a single step.

An ATP system is formed when two water-soluble polymers, such as poly (ethylene glycol) (PEG) and dextran, or a polymer and a salt are dissolved in water beyond a critical concentration at which two immiscible phases form. Both phases can enhance protein stability [9]. The equilibrium partitioning of a protein in an ATP system depends on protein surface properties and the physicochemical properties of the two phases. Surface properties include surface net charge and hydrophobicity; phase system properties include polymer molecular weight (MW) and concentration, type of phase forming salt, salt concentration, and pH [7, 9]. PEG - dextran and PEG - salt systems have been widely studied for protein separation; PEG - salt systems have advantages of lower viscosity and lower cost [10].

To date, ATP partitioning has been used to fractionate gluten from wheat [11], purify peroxidase from soy [12], and separate tobacco leaf proteins [13]. To develop a suitable ATP separation method for recombinant protein from corn, the partitioning behavior of native corn proteins from different tissues and the relation between protein partitioning and ATP system parameters need to be known.

The effect of procedure on corn protein partitioning, NaCl addition and phase ratio on model protein-lysozyme purification will be discussed in this paper.

1.1. Definition of parameters in ATP systems

The partition coefficient ($K_i$) is the ratio of concentrations in each phase:

$$K_i = \frac{C_{i,\text{top}}}{C_{i,\text{bottom}}}$$

The extent to which an ATP system to separate target protein from host protein in a single stage is the selectivity ($\alpha$):

$$\alpha = \frac{K_{\text{target}}}{K_{\text{host}}}$$

The purification factor ($PF$) for recovery in the top phase is defined as the ratio of final specific activity ($SA$) to initial $SA$

$$PF = \frac{SA_{\text{top}}}{SA_{\text{initial}}}$$

Phase ratio ($\phi$) is the relative volume of the two phases:

$$\phi = \frac{V_{\text{top}}}{V_{\text{bottom}}}$$

where $V_i$ is the volume of phase $i$.

Total recovery ($R_T$) of protein is defined as the ratio of protein $i$ dissolved in top and bottom phases to that extractable in buffer without phase-forming solutes:

$$R_T = \frac{C_{i,\text{top}} \times V_{\text{top}} + C_{i,\text{bottom}} \times V_{\text{bottom}}}{M_i}$$
Yield (Y) of protein is defined as recovery in the top phase:

\[ Y_i = \frac{C_{i}^{\text{top}} \times V_{i}^{\text{top}}}{M_i} \]

(6)

2. Materials and methods

2.1. Materials

Yellow dent corn endosperm commercially degenerated and milled to a particle size range from 0.33 to 0.85 mm was supplied by Lauhoff Grain Co. (Danville, IL), defatted corn germ milled to a particle size range from 0.03 to 0.15 mm was supplied by ProdiGene Inc (College Station, TX). PEG 3350, hen egg white lysozyme (L7001), and *Micrococcus lysodeikticus* (M3770) were purchased from Sigma-Aldrich Corp. (St. Louis, MO, USA). Coomassie Plus® protein assay reagent kit was purchased from Pierce Biotechnology (Rockford, IL, USA). ACS certified grade sodium phosphate monobasic, sodium sulfate, hydrochloric acid and sodium hydroxide were from Fischer Scientific (Pittsburgh, PA, USA).

2.2. Methods

ATP systems PEG 3350 (15.7%) – Na₂SO₄ used in this paper have been reported [9, 14-17]. Two procedures were used to prepare the ATP systems: (1) clarified extract partitioning: mixing clarified extract, PEG stock solution, salt stock solution or solid salt; (2) integrated procedure: corn solids added directly to preformed ATP system for integration of extraction and partitioning.

2.2.1. Preparation of extracts and stock solutions

Corn endosperm or germ solids were added by weight to 50 mM sodium phosphate at the desired pH (pH 4, 7, or 9) at a level of 10 g solids/50 ml buffer. The slurry was stirred for 1 h by magnetic stir bar and pH controlled by addition of 1 M NaOH or 1 M HCl. To prepare clarified extracts, the slurry was centrifuged (3000 g, 22°C, 30 min), decanted and the supernatant filtered through a 0.45 μm μStar cellulose acetate syringe filter (Costar Corp., Corning NY).

Na₂SO₄ stock (23%) and PEG stock (50%) solutions were prepared by dissolving the required amount of solute in 50 mM sodium phosphate, pH 7 buffer.

Lysozyme was dissolved in 66 mM potassium phosphate, pH 6.24, as recommend by the supplier, to prepare the 5 mg protein/ml stock solution.

2.2.2. Partitioning

The sample loadings were 0.1 g endosperm or 0.01 g germ solids for the integrated procedure, while the clarified extracts (prepared at 1:5 w/v solid:buffer ratios) are added in volumes corresponding to initial solids equivalent to those added in the integrated procedure. The integrated procedure with added lysozyme was also carried out in PEG 3350 - Na₂SO₄ systems, examining the effects of NaCl addition, and phase ratio.

ATP aliquots (1.5 g) were prepared by combining appropriate stocks of phase components, extracts or corn solids (germ 0.01 g/g ATP aliquots or endosperm 0.1 g/g ATP aliquots), any added NaCl and diluted to final mass with extract buffer. For the integrated procedure, the mass of corn solids was not counted toward the 1.5 g total mass.

The mixtures were thoroughly mixed by vortexing for 30 seconds, equilibrated at room temperature (22°C) for 1 hour with mixing by tumbling, and centrifuged (3000 g, 22°C, 20 min), to expedite the phase separation. After recording the volume of each phase, the top phase was carefully removed by transfer pipette and the bottom phase by piercing the centrifuge tube with a syringe; the region near the interface and the solids layer in tube bottom was left in the tube to
avoid contamination of the phase samples. The total protein and lysozyme concentrations in samples from each phase were measured as described below.

For the lysozyme-spiked samples, a large volume of ATP system (PEG 3350 - Na₂SO₄ with added NaCl) was prepared and the two phases separated. The individual phases were combined in the desired phase ratio with the corn solids (germ 0.01 g/g ATP aliquots or endosperm 0.1g/g ATP aliquots) and lysozyme stock (providing 22.5 μg lysozyme) to prepare 1.5 g aliquots for partitioning.

2.2.3. Assay methods

Protein concentration was determined by Coomassie Plus® protein assay reagent kit (Pierce Rockford, IL), using bovine serum albumin (BSA) as standard. The appropriate blank aqueous system phase was employed to correct for minor interference from salt and PEG. The activity assays for lysozyme followed the standard protocol for enzymatic assay of lysozyme from Sigma-Aldrich [18]; assay blanks were phases from integrated partitioning of corn solids with no added lysozyme. All partitioning experiments were replicated three times and each replicate assayed twice.

3. Results and discussion

3.1. Native corn protein partitioning

3.1.1. Protein extraction from corn endosperm and corn germ

Fig. 1 shows that the extraction of total proteins (mg protein/g solids) from both germ and endosperm increases as pH increases from 4 to 9. Initially this is the result of moving away from the overall isoelectric point [19] and eventually to greater release of the glutelins fraction [6]. Furthermore, the amount of protein extracted from germ is much higher than from endosperm. Hence, when corn will be used for recombinant protein production, a recombinant protein stable at low pH (pH 4) could be more easily purified because the level of contaminating protein is relatively low. Further, expression in endosperm avoids most soluble host protein – a clear advantage assuming similar expression levels can be achieved with this targeting.

3.1.2. Integrated extraction and partitioning

In the PEG - sulfate system (Table 1), for endosperm, the amount of extracted protein is ca. 30% less in the integrated procedure and, for germ, it is ca. 50% less. Therefore, the lower K's in the integrated procedure could result from either lower protein concentration (although these are still in the range that independence could be expected) or different protein composition. The lower level extracted proteins in the integrated procedure would result in less contamination in extracts of recombinant proteins whose solubility was not limited by the phase components.

3.2. Optimization of lysozyme purification from corn extracts

3.2.1. NaCl addition

NaCl addition dramatically enhanced partitioning of lysozyme along with selectivity (Fig 2 and Table 2). The K of lysozyme increased from 0.37 to above 100 with 4.5 wt% NaCl addition, whereas K of corn protein only increased about two-fold. Because lysozyme partitioned totally to the top phase at 4.5 wt% NaCl, more NaCl addition only pushed more corn protein to the top phase and decreased the purification factor. In addition, the purification factor of lysozyme from germ is higher than from endosperm due to germ's lower partitioning to the top phase.

3.2.2. Phase ratio

Having fixed the nature of top and bottom phases to provide favorable selectivity, product recovery and overall purification factor can still be manipulated by controlling the relative volumes of top and bottom phases (i.e. phase ratio). In principle, selectivity relative to
individual proteins should not change; however, here we are looking at selectivity relative to a mixture of native proteins where changes in phase ratio can lead to nonuniform losses from precipitation at the interface so that the apparent $K$ of native proteins and selectivity would also change. The integrated partitioning results of lysozyme with corn solids in PEG 3350 - Na$_2$SO$_4$ system with 4.5 wt% NaCl at different phase ratios (0.71, 0.44 and 0.15) are shown in Figs. 3. The $K$ (Fig. 3a) and additionally the extraction of corn protein decreased with increasing phase ratio, while lysozyme partitioned essentially completely to the top phase for all phase ratios. As a result, the purification factor (Fig. 3b) while maintaining complete recovery.

4. Conclusion

The amount of protein extracted from endosperm was much less than that from germ; in both cases amount of extracted protein increased with pH. Extracted corn protein (at pH 7) favored the bottom phase in PEG 3350 (15.7 wt%) -Na$_2$SO$_4$ (8.9 wt%) ATP systems. $K$ of endosperm protein was higher than that of germ protein. In addition $K$ and extraction of corn proteins decreased in integrated procedures.

NaCl addition enhanced the selective recovery of lysozyme in the top phase of PEG 3350 (15.7 wt%) -Na$_2$SO$_4$ (8.9 wt%). At 4.5 wt% NaCl, selectivity for lysozyme in the top phase relative to corn fraction proteins was 134 and 616 for endosperm and germ respectively, with essentially complete recovery in the top phase. For a phase ratio of 0.15, the purification factor for lysozyme from germ protein was 14.

Acknowledgments

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References


Table 1. Protein partitioning in extraction/partitioning procedures in PEG 3350 (15.7%) - Na₂SO₄ (8.9%).

<table>
<thead>
<tr>
<th>Corn fraction</th>
<th>Procedure ⁴</th>
<th>K</th>
<th>Protein recovered in two phases ⁵</th>
<th>Comments</th>
<th>Location of corn solids</th>
</tr>
</thead>
<tbody>
<tr>
<td>Endosperm</td>
<td>Clarified</td>
<td>0.8±0.17</td>
<td>153±11</td>
<td>Yes</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Integrated</td>
<td>0.26±0.05</td>
<td>100±6</td>
<td>Yes</td>
<td>Tube bottom</td>
</tr>
<tr>
<td>Germ</td>
<td>Clarified</td>
<td>0.38±0.13</td>
<td>610±54</td>
<td>Yes</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Integrated</td>
<td>0.12±0.01</td>
<td>306±70</td>
<td></td>
<td>Coarse at tube bottom and fine at interface</td>
</tr>
</tbody>
</table>

¹ Sample loading 0.1 g endosperm or 0.01 g germ solids was added to buffer before clarification or directly to ATP system per g final ATP system.
² Calculated from measured concentrations and volumes of the two phases. For the integrated procedure, the accuracy is limited because particles at the interface and tube bottom make the accurate volume determination difficult. Protein that may have precipitated at the interface is not accounted for.
³ For the clarified extract protein loading is calculated from the protein concentration of clarified extract and the volume of that extract added to the system.
⁴ ± ranges are 95% confidence levels.
**Table 2.** Lysozyme purification performance.
Integrated extraction and partitioning of lysozyme and corn fraction proteins in PEG 3350 (15.7%) - Na$_2$SO$_4$ (8.9%) system with added NaCl. Solids loading of 0.10 g endosperm or 0.01 g germ combined with 15 μg lysozyme/g ATP system.

<table>
<thead>
<tr>
<th>Corn fraction</th>
<th>[NaCl]/wt%</th>
<th>Phase ratio</th>
<th>Corn protein yield in top phase (%)$^a$</th>
<th>Purification factor</th>
<th>Selectivity in top phase</th>
<th>Lysozyme yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Endosperm</td>
<td>0.00</td>
<td>1</td>
<td>9±2$^b$</td>
<td>2.52±0.23</td>
<td>±0.27</td>
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</tr>
<tr>
<td>Endosperm</td>
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<td>0.71</td>
<td>12±5</td>
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<td>151±33</td>
<td>108±5</td>
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<tr>
<td>Endosperm</td>
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<td>0.71</td>
<td>20±5</td>
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<td>140±14</td>
<td>105±5</td>
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<tr>
<td>Germ</td>
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<td>3.37±0.55</td>
<td>3.04±0.49</td>
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<tr>
<td>Germ</td>
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<td>8.43±0.60</td>
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<tr>
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<td>23±3</td>
<td>4.63±0.73</td>
<td>221±37</td>
<td>103±5</td>
</tr>
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</table>

$^a$ Corn protein yield based on assuming that the protein available from the solids is the protein that would be extracted by 50 mM phosphate buffer at pH 7 with solid: buffer ratio of 1 g: 5 ml and that the amount recovered is that resulting from complete recovery of the top phase.

$^b$ ± range is the 95% confidence level.

---

**Fig. 1.** pH dependence of protein extraction. Germ values was 10× higher than shown on the axis. Conditions: 1 g solids: 5 ml buffer (50 mM sodium phosphate)
Fig. 2. NaCl effect on protein partitioning for the integrated extraction and partitioning of lysozyme and corn fraction proteins in the PEG 3350 - Na$_2$SO$_4$ phase system with added NaCl. 0.10 g endosperm or 0.01 g germ solids combined with 15 µg lysozyme/g ATP system.
Fig. 3. Phase ratio influence on protein partitioning for the integrated extraction and partitioning of lysozyme and corn fraction proteins in the PEG 3350 - Na₂SO₄ phase system with 4.5 wt% NaCl. 0.10 g endosperm or 0.01 g germ solids combined with 15 μg lysozyme/g ATP system. (a) corn protein partitioning; (b) purification factor.
Lipase Catalyzed Esterification of Geraniol in Ionic Liquid 
[bmim]PF$_6$ mediated by pervaporation

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Abstract

The use of non-aqueous media has been shown to be effective for carrying out enzymatically catalyzed reactions in which the reactants are not water-soluble or in which water is a byproduct that limits conversion. Organic solvents are commonly used for this purpose, yet, they are potentially environmentally damaging. Ionic liquids, organic salts that are liquid at room temperature, are a potentially useful alternative. In this work, the direct esterification of geraniol with acetic acid in [bmim]PF$_6$ has been investigated. Immobilized Candida Antarctica lipase B has been used to catalyze the reaction. Pervaporation was used to carry out reactions at fixed water activities by removing the water produced. Significant enzymatic activity is observed in [bmim]PF$_6$ although the reaction rate was slower than in organic solvents. No significant differences in reaction rate were found in the interval of water activity tested. The equilibrium constant for the reaction was found experimentally.

Introduction

Enzymes have shown to be active in media other than water and it has been possible to carry out reactions where water is one of the reaction products, or using non-water soluble substrates [Klibanov, 1997]. Particularly, many enzymes show enhanced selectivity, stability and selectivity in organic solvents. The main chemicals that are produced using this technique are fragrances, flavors and specialized pharmaceuticals [Krishna, 2002]. However, since most organic solvents are volatile they have a detrimental effect on the environment and it is necessary to find synthetic techniques for reducing the level of emissions and the environmental harmfulness [Nara et al., 2002].

Besides organic solvents, supercritical fluids, gases and ionic liquids has been used as non conventional media for enzymatic reactions [Krishna, 2002]. The last group of solvents has gained much attention in the last years because of their novel properties. Room Temperature Ionic Liquids or simply “Ionic Liquids (IL)” are salts with melting points below 100 ºC. Because of their ionic nature, ionic liquids have negligible vapor pressure and are thermally stable [Huddleston et al., 2001]. They are attractive to replace organic solvents in chemical processes since it will eliminate volatile organic compound (VOC) emissions. Most ionic liquids are composed of one organic cation and one inorganic or organic anion. It has been shown that the properties of the ionic liquids can be adjusted by selecting proper cation – anion combinations [Huddleston et al., 2001].

Theoretically, it is possible to design a particular ionic liquid for any specific application [Nara et al., 2002]. Different cation-anion combinations are shown in Figure 1.

![Figure 1. Typical anion-cation combinations in ionic liquids. Water solubility changes as different pairs are selected.](image)

Ionic liquids are good solvents; they are able to dissolve polar and non-polar organic components. They also have a wide liquid range (usually greater than 300 °C) so are suitable to carry out typical liquid phase reactions [Seddon et al., 2000]. Although no studies have been reported on the effects of increasing the IL presence in the environment, they are generally considered environmentally friendly because they are stable towards air and water [Seddon et al., 2002].

In this work we carry out the direct esterification of geraniol with acetic acid in ionic liquid 1-butyl-3-methyl imidazolium hexafluorophosphate, [bmim]PF₆, catalyzed by immobilized enzyme *Candida antarctica* lipase B (CALB) Novozym® 435 (scheme 1).

\[
\text{Geraniol} + \text{Acetic acid} \rightleftharpoons \text{Geranyl Acetate} + \text{Water}
\]

Scheme 1: Direct esterification of geraniol to produce geranyl acetate and water

Geranyl acetate is an important chemical used extensively in fragrance and flavor industries [Peres et al., 2003].
*Water activity*

It has been shown that water content influences the enzymatic activity in non aqueous media. [Peres et al., 2003; Ducret et al., 1998; Berberich et al., 2003]. Water acts a lubricant that allows the enzyme to exhibit the conformational mobility required for catalysis [Klivanov, 1997; Halling, 1994]. The thermodynamic water activity has been shown to be the best guide to the amount of water in the microenvironment in which the kinetic process occurs [Halling, 1994]. At equilibrium, thermodynamic water activity will be equal between the environment that surrounds the enzyme and the bulk phase of the solvent, so it can be measured in the bulk of the fluid in order to account for water effects in the vicinity of the enzyme. The activity of water in a solution is expressed as [Model and Tester, 1994]

\[ a_w = \gamma_w x_w \]  \hspace{1cm} (1)

Where \( a_w \) is the water activity, \( x_w \) is the molar fraction of water in solution and \( \gamma_w \) is the activity coefficient of water. At the vapor-liquid interface the equilibrium condition is expressed as

\[ a_w f_w^o = \phi_w y_w P \]  \hspace{1cm} (2)

Where \( f_w^o \) represents the fugacity of water in some reference standard state and \( \phi_w \) is the fugacity coefficient of water in the vapor phase. If the vapor phase behaves ideally then \( \phi_w = 1 \). Choosing pure water as standard state \( f_w^o = P_w^{sat} \), and assuming that water is the only component that has a significant vapor pressure in solution \( (y_w \approx 1) \), then

\[ a_w = \frac{P}{P_w^{sat}} \text{ or } \gamma_w = \frac{P}{P_w^{sat} x_w} \]  \hspace{1cm} (3)

Anthony et al. [2001] estimated the activity coefficients for water dissolved in [bmim]PF₆ using liquid-liquid equilibrium data. They also assumed Henry's law behavior \( (\gamma_w \text{ independent of the concentration}) \) for the equilibrium between the vapor and the liquid phase in order to calculate activity coefficients; from these data \( \gamma_w (30 \degree C) = 4.88 \). Berberich et al. [2003] measured the water activity as a function of the water content by equilibration with salt hydrate pairs, and compared their results with Anthony's predictions. There was a good agreement between the estimated \( a_w \) and the experimental results.

*Pervaporation*

Because of its influence on the catalytic activity of the enzymes, different methods have been used to control and measure the water activity in enzymatic reactions [Kaar et al., 2003; Feng et al., 1996; Bartling et al., 2001]. This becomes more important for the case in which water is produced during the reaction. Common methods are: (i) add pre-equilibrated silica gel to the reaction mixture, (ii) use of salt solutions to equilibrate water
activity between the solvent and the salt, (iii) dry the head space above the solvent, (iv) add solid salt hydrates to the reaction mixture and (v) equilibrate the head space in the reactor with saturated salt solutions [Halling, 1994].

Even though those methods are effective, in most of them is necessary to contaminate the mixture by adding an external agent, making the recovery and purification of the enzyme and the solvent more difficult. Beside this, when solid salts or salt solutions are used, the water activity values that can be set are restricted to some fixed values [Berberich et al. 2003]. Pervaporation has been used to remove volatile solutes selectively from ionic liquids and other solvents [Schaffer et al., 2001; Gubicza et al., 2003; Bartling et al., 2001]. This separation method overcomes the drawbacks of the salt equilibrations since it is not necessary to add extraneous chemicals to the system and theoretically any water content can be set. Pervaporation has been successfully used to precisely control the thermodynamic water activity in the esterification of geraniol in hexane [Kang et al., 2004]. The ability to maintain precise control of water activity even in the presence of water production demonstrates the power of the pervaporation technique. Achieving similar results when IL is used as solvent should be readily possible as the IL has negligible vapor pressure and should not be evaporated. Indeed, some preliminary works have shown the potential of this technique in controlling water activity for esterification reactions in ionic liquids [Gubicza et al., 2003].

Pervaporation is a separation technique in which a multicomponent liquid is passed across a membrane that preferentially permeates one of the components. Typically, a liquid feed contacts one side of a polymeric membrane and the permeate is removed as vapor on the other side [Huang, 1991]. Driving force for transport through the membrane is the difference in vapor pressure between the liquid feed and the permeated vapor. The flux through the membrane is a function of the vapor pressure difference, \((P_{wo} - P_{w1})\), the thickness of the membrane, \(l\), and the permeability coefficient of water in the membrane \(P_w^G\) [Baker, 2000]

\[
J_w = \frac{P_w^G}{l} (P_{wo} - P_{w1}) \quad (5)
\]

Pervaporation processes can be carried out using low temperatures and pressures so they can be applied without degrading the catalyst or interfering with the reaction system.

Background

Different results have been published on the use of lipases to catalyze esterification reactions in ionic liquids. Kaar et al. [2003] studied the impact of different ionic liquids on the reaction properties of lipases. Transesterification of methyl methacrylate was used as a model reaction. Most of the ionic liquids tested (water soluble and non-soluble) showed no activity for free or immobilized enzymes. However, free CALB lipase was 1.5 times more active in \([\text{bmim}]\text{PF}_6\) than in hexane although no activity was reported for immobilized Candida Antarctica lipase B Novozym® 435. Gubicza et al. [2003] carried out the enantioselective esterification of (R,S)-2-chloropropanoic acid with 1 butan-1-ol, using free Candida Rugosa lipase. Although the enantioselective properties of the
enzyme were not studied, the activity of the enzyme in [bmim]PF$_6$ was comparable with that in hexane. Lozano et al. [2001] carried out the butyl butyrate synthesis catalyzed by free Candida Antarctica lipase B using four different ionic liquids. All of the ionic liquids tested were shown to be suitable media for the transesterification reaction. The enzyme activity in [bmim]PF$_6$ was about twice that in hexane. Itoh et al. [2001] have reported a 5-phenyl-1-penten-3-ol conversion of 47% after 5 h for the transesterification reaction with vinyl acetate using Novozym® 435 as catalyst. Husum et al. [2001] reported 90% butyric acid conversion after 1 h for the esterification reaction with a simple alcohol. Novozym® 435 was used as catalyst. Different results obtained by several groups could be attributed to differences in ionic liquids properties; many of them were synthesized in the laboratory, as has been reported many residual solutes from the synthesis process have a strong influence on the properties of the ionic liquids [Seddon et al., 2000].

Although no data was found on the geranyl acetate synthesis in ionic liquids, its production has been reported using organic solvents as reaction media. Chulalaksananukul et al. [1992] carried out the transesterification of several acetates to produce geranyl acetate in hexane using Lipase from M miehei. It was shown that there is an optimum level of water content (measured in the solid support of the immobilized enzyme) for which the initial rate has a maximum. Kinetic data obtained by experimentation was fitted using a ping pong bi bi mechanism with substrate inhibition. Akoh and Claon [1994] showed that Candida antarctica lipases SP382 and SP435 were able to catalyze the direct esterification of geraniol to produce geranyl acetate in n-hexane. They studied the influence of the amount of enzyme used, temperature and water content of the mixture on the reaction rate. Huang and Chang [1999] carried out a kinetic study on the direct esterification of geraniol to geranyl acetate in isooctane using a surface coated lipase from Candida cylindracea. A ping pong bi bi model with competitive and dead-end inhibition by acetic acid was fitted. It was also shown that the water content has a strong influence on the reaction rate. Although these studies addressed the influence of water in the final conversion no water control was reported. Bartling et al. [2001] used pervaporation to remove water from the reaction mixture for the esterification of geraniol with acetic acid in hexane. Immobilized enzyme Novozym® 435 was used. The conversion for the reaction mediated by pervaporation was close to 100% and the reaction rate was 1.5 times greater than with no water removal. Subsequently Kang et al. [2004] developed an online sensor that allowed fast (<10s) measurement of the thermodynamic water activity in the multicomponent system. Using this sensor and a pervaporation membrane system, the thermodynamic water activity of the reaction mixture was precisely controlled. The results demonstrated a maximum reaction rate at $a_w \approx 0.1$ which was twice that at other conditions.

**Experimental**

**Chemicals.** The reaction was conducted in ionic liquid [bmim]PF$_6$ as solvent (Solvent innovation, Cl<1000ppm, >99% pure). Geraniol (98% pure) and geranyl acetate (98% pure) were obtained from Sigma. Glacial acetic acid (>99.7% pure, optima), acetone (>99.6% pure), benzyl alcohol (>99.8% pure) and hexane (>99.9% of saturated hydrocarbons) were obtained from Fisher Scientific. Cellulose acetate was obtained form
Celanese Acetate. The averaged degree of substitution was 2.91 [Variankaval et al., 2003] Chemicals were used as received.

**Enzyme.** A non specific thermostable lipase from *Candida antarctica* as a commercial preparation, Novozym 435® (Novozymes inc.) was used. This enzyme is classified as a tryacylglycerol hydrolase (E.C. 3.1.1.3). The ester synthesis activity of the enzyme expressed in Propyl Laurate per gram (PLU/g) is about 10,000 PLU/g. The enzyme preparation consists of the enzyme immobilized on a macroporous acrylic resin consisting of spherical particles with diameter in the range of 0.3 to 0.9 mm (bulk density: 430 kg/m$^3$, water content < 3% w/w) [Novozyme, 2003]. Enzyme preparation was purchased from Sigma and stored at 4 °C. Enzyme preparation was used as received.

**Geraniol conversion and water content:** To measure the geraniol conversion the following method was used: 0.25 ml of the ionic liquid that contains geraniol and geranyl acetate were mixed with 1.5 ml of acetone in a 4 ml screw capped vial. The resulting mixture was analyzed by gas chromatography. To carry out the GC analysis 0.5 µl aliquots were injected into a Varian 3800 gas chromatograph (FID detector, capillary column DB-5, 30 m length, 0.25 mm i.d., isothermal 120 °C; J&W Scientific). Injection temperature and pressure were 250 °C and 30 psia. An injection split ratio of 1/200 was used. Retention times at these conditions were 1.9 min and 3.3 min for geraniol, and geranyl acetate respectively. The conversion was correlated with the ratio between geraniol and geranyl acetate areas and the respective response factors. Response factors were found by calibration with known concentration solutions. Water content was measured by KF titration (Denver Instruments model 270). 0.25 ml aliquots were withdrawn periodically from the reactor; and used to carry out the KF titration.

**Membrane fabrication and characterization.** Polymer/ceramic composite membranes were fabricated by casting a cellulose acetate-acetone solution (1.5% weight, filtered using a 1.2-µm syringe filter), onto an alumina ceramic membrane (Whatman Anodisc, pore size: 0.02µm, diameter: 47 mm). The support was placed on a flat plate and then using a stainless steel ring, a solution column of 4 mm was filled upon the support surface. The solvent was allowed to evaporate overnight under a nitrogen-acetone atmosphere. Then the membrane was separated from the ring using water and dried in the pervaporation cell for four hours.

The quality of the composite membranes was determined by permeation tests with nitrogen and carbon dioxide [Koros, 1977]. The composite membrane ideal CO$_2$/N$_2$ selectivity (20.2) is in the reasonably good agreement with the value of 25.8 reported for cellulose acetate by Li et al. [1995]. Using the Li et al. values for permeability of nitrogen the polymer layer thickness was estimated to be 1.4 ± 0.65 µm.

**Reaction Test.** Reactor and other hardware were washed with acetone and dried to 105 °C before every experimental run. The ionic liquid was placed into the reactor and heated to the reaction temperature (30 °C). Then, geraniol (0.1mol/L), acetic acid (0.1mol/L) and water (initial content) were added. Total reaction volume was 100 ml. After the reactants were added, the reactor was sealed and kept at 30 °C using a water
bath. After 24 hours geraniol was completely dissolved and the reaction was started by adding the enzyme (0.1 g of enzyme preparation /g reactants) to the reactor. The reactant mixture was then pumped (FMI lab pump, model QV) across the membrane unit (Millipore Stainless steel filter holder, filtration area: 13.8 cm²). All feed side circulation system was built using 1/8’ tubing and fittings (Swagelock, Inc.). A Tyler 180 stainless steel mesh was used in order to retain the catalyst inside the reactor. The vacuum system was built using ¼’ VCR vacuum fittings and tubing (Swagelock, Inc.). The flux increasing was monitored using a pressure transducer (MKS Instruments, type 122A. Range = 10 Torr). Vacuum was achieved by using a pump (Edwards, RV5). Temperature was set and controlled at 30 °C for all runs (Omega temperature controller CN1A-TC; Glass Col heating tape, 3 Amp). For the case were not water was removed the membrane unit was bypassed. The experimental set up used is shown in figure 2. All hardware was washed with acetone and dried to 105 °C before each run. 0.5 ml aliquots were withdrawn periodically from the system and the geraniol and water contents in the ionic liquid were then measured.

Results and discussion

Pervaporation studies

The permeated flux through the membrane was correlated as a function of the water activity. The calibration curve is shown in figure 3. Water activities were estimated by using equation (1) and the data reported by Anthony et al. [2001] and Bereberich et al [2003]. Comparison with the literature shows that the flux, at the same water content, is approximately 6 times higher than the reported by Schaffer et al. [2001] for the pervaporation of water from [bmim]PF₆ through several polymeric membranes. This difference can be attributed to differences in the polymers water permeability and the thickness of the polymer layer.
Because of the large variation in the flux, the maximum achievable pressure at the permeate side of the membrane, with the valve to the vacuum pump totally open, changed from 0.6 to 5 mmHg. If the permeability coefficient is independent of the water content, one would expect a linear relationship between \(a_w\) and water flux [Baker, 2000]. Analysis of figure 3 clearly indicates that no such linear relationship exists. This suggests that the cellulose acetate membrane employed has a concentration dependent water permeability. This kind of behavior has been previously observed for cellulose acetate [Huang, 1991].

![Graph](image.png)

Figure 3. Water permeation flux vs. water activity. (•) experimental, (—) exponential regression, (---) 95% prediction interval.

Esterification reactions

The geraniol esterification was carried out in [bmim]PF₆. Reaction conditions are consistent with prior work in hexane: Temperature = 30°C, equimolar concentration of reactants, alcohol concentration = 0.1 mol/L [Bartling et al., 2001]. Pervaporation was used to control the water activity in the mixture during the reaction. Several runs were performed at different water activities in order to assess the water influence on the reaction rate. These conditions were: (i) no water removal; initial \(a_w\) = 0.05, (ii) \(a_w\) = 0.10; constant, (iii) \(a_w\) = 0.20; constant, (iv) \(a_w\) = from 0.37 to 0.14; the water activity was reduced during the reaction and (v) \(a_w\) = 0.58; constant. Different flow rates across the pervaporation unit were used to remove the amount of water needed in each case. Calculation of water activity was made assuming no interaction of water with the other components in the mixture and the data of Anthony et al. [2001] and Berberich et al. [2003]. Time-conversion profile for each case is shown in figure 4. No significant differences on reaction rates were found in the interval of water activities studied. As was mentioned earlier Kang et al. [2004] showed the existence of a maximum in the reaction rate at an activity of 0.1 in hexane. Theoretically, this optimum should remain unchanged for other solvents since the microenvironment around the enzyme doesn’t change [Halling, 1994]. Yet the effect of the solvent on the enzyme should be considered. The solvent may alter the conformation of the enzyme, the local concentration of the substrates and the ability of the enzyme to change conformation [Klivanov, 1997]. Other
water activities should be considered in order to find the optimum water activity for enzymatic catalysis and other effects tested before definite conclusions can be drawn.

![Figure 4](image-url)

Figure 4. Conversion-time profiles for reactions for several water activities. Geraniol and acetic acid: 0.1 mol/L, 30 °C. (●) no water removal, (●) $a_w=0.10$, (×) $a_w=0.20$, (▲) $a_w=0.38$ to 0.14, and (○)$a_w=0.58$.

As mentioned earlier, no previous studies for the direct esterification of geraniol in ionic liquids were found. In this regards, this study contributes to broaden the spectrum of reactions that can be applied in ionic liquids. Comparison of these results with previously reported data in other solvents shows that in general the enzyme was less active in this ionic liquid than it was in organic solvents [Akoh et al., 1994; Bartling et al., 2001; Kang et al., 2004] and in supercritical fluids [Peres et al., 2003]. At constant $a_w=0.1$, the initial reaction rate in hexane [Kang et al., 2004] was 100 times-fold that in ionic liquid. However, although this is the optimum water activity for enzymatic activity in hexane it may not be the optimum for ionic liquids. Indeed Berberich et al. [2003] reported that esterifications in ionic liquids with very low water contents had reaction rates more than 50-times those achieved at high water levels. A more consequential comparison would be made at optimum water activities.

During the reactions pervaporation was effectively used to control water content and in this way the water activity. The water content profile for reaction (ii) $a_w=0.10$ is shown in figure 5. Although permeated was not recovered, previous studies [Bartling et al., 2001] have shown that cellulose acetate films are highly selective to water. Since the ionic liquid has no vapor pressure water is considered the only species dissolved in the polymeric film. The control was carried out by changing the flux across the membrane unit. The water activity was controlled within a 95% confidence interval of ±0.006 units.
Figure 5. Water content-time profile for reaction (ii) $a_w=0.10$. (•) experimental results. (—) average (---) 95% confidence interval.

Equilibrium conversion

Although [bmim]PF$_6$ is often classified as an "hydrophobic" ionic liquid [Anthony et al., 2001; Seddon et al., 2000] it has a water solubility of 23000 ppm [Wong et al. 2002; Anthony et al., 2001], or about 1.8 mol/L. At the conditions used (0.1 mol/L for both reactants) even at low water activities (for example at $a_w=0.1$, $C_w\approx0.18$ mol/L) water will have a marked influence on the equilibrium conversion. This important fact means that a high water activity optimum for enzymatic activity would not be necessarily the optimum for ester yield, since at high water activities the equilibrium conversion will be low. The equilibrium conversion is also influenced by the nature of the solvents. Partitioning of the substrates between phases is different for different solvents [Halling, 1990; Valivety et al., 1991]. For cases (ii) $a_w=0.10$, and (iii) $a_w=0.19$, reactions were carried out at fixed water activities until the equilibrium conversion was reached. This condition was characterized by the plunge of the reaction rate to zero. Conversion profiles are shown in figure 6. Equilibrium constant calculations based on thermodynamic activities are usually difficult since ester, alcohol and acid activity coefficients are no known. Regarding this, the equilibrium constant based on concentrations was calculated in order to compare it to previous data reported. The equilibrium constant then was calculated using

$$K_c = \frac{[Ester][Water]}{[Acid][Alcohol]}_{equilibrium}$$

$$K_c = 2.614 \pm 0.165$$

The calculated value is about 100 times lower than the reported by Bartling et al. in hexane [2001]. This indicates the different nature of the interactions between substrates and the solvent in the ionic liquid and in hexane. Water activity also influences the partition of the substrates between the phases [Halling, 1994]. Valivety et al. [1991]
correlated the equilibrium constant for the esterification of dodecanol in a number of organic solvents. Results shown here are in the same order of magnitude of Valivety's data.

Figure 6. Conversion-time profiles for reactions at different water activities. Geraniol and acetic acid: 0.1 mol/L. 30 °C.

Conclusions

This work has proven that Novozym® 435 is effective in catalyzing the esterification of geraniol with acetic acid when ionic liquid [bmim]PF₆ is used as solvent. It helps to support the conclusion that lipases are active in these solvents. Since no previous data has been reported for this system, this work also helps to broaden the spectrum of enzyme catalyzed reactions that can be carried out in ionic liquids. Pervaporation was used effectively to carry out reactions at constant water activities. Although different water activities were tested no significant differences were shown in reaction rates. However, the interval of water activities was limited and other factors can affect the reaction rate, and further investigations would be necessary to clarify this point. Reaction rate was lower than in other non aqueous solvents reported. The concentration equilibrium constant was calculated for the reaction and showed to be lower than the previously reported for hexane.

Acknowledgment

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A Novel Class of Polyanhydrides with Tailored Erosion Mechanisms

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Abstract

Biodegradable polymers have been used effectively as drug delivery devices. Specifically, polyanhydrides, which are surface erodible polymers, have shown excellent performance as drug carriers. These polymers accomplish the dual objective of modulating drug release while simultaneously protecting the drug for a prolonged period. Their hydrophobic nature prevents water penetration into the bulk, thus eliminating water-induced covalent aggregation of proteins. However, protein inactivation by non-covalent aggregation remains an issue. Previous studies have indicated that amphiphilic polymers offer a gentle environment for proteins. We propose that incorporation of hydrophilic entities, such as oligomeric ethylene glycol, into anhydride monomers may prevent inactivation by both covalent and non-covalent aggregation mechanisms. Using these monomers, we have synthesized copolymers based on the anhydrides monomers 1,6-bis(p-carboxyphenoxy)hexane (CPH) and 1,8-bis(p-carboxyphenoxy)-3,6-dioxaoctane (CPTEG), which contains oligomeric ethylene glycol moieties. The studies demonstrate that by increasing the CPH content in CPTEG:CPH copolymers, the erosion of the system can be tailored from a bulk-eroding to a surface-eroding mechanism.

Introduction

Polyanhydrides are a class of surface bioerodible polymers that have been extensively used as carriers for controlled drug delivery. The promising characteristics of this class of polymers have led to extensive research on the chemistry, microstructure, and biocompatibility in the last two decades. The biocompatibility studies, both in vitro and in vivo, have shown that these biomaterials degrade into non-mutagenic and non-cytotoxic products. The surface erosion mechanism exhibited by these polymers leads to a controlled release profile with a predictable hydrolytic degradation, which can range from days to months, depending on the polymer chemistry.

The overall goal of our research is to engineer biomaterials suitable for the stabilization and sustained release of proteins. Currently there are several hundred investigational new protein drugs that have not been approved by the United States Food and Drug Administration (FDA) due to the lack of a suitable delivery device. Finding the appropriate carrier for proteins is a complex task that involves a fundamental understanding of the inactivation mechanisms of the protein, the interactions between the protein and the carrier, and the carrier chemistry.

It has been suggested that the use of carriers containing both hydrophobic and hydrophilic entities may provide a gentler environment for proteins. Previous research has demonstrated that polyanhydrides, which are highly hydrophobic, can prevent covalent aggregation by reducing the water penetration into the core. However, strong hydrophobic interactions between the polymer and the protein may lead to non-covalent aggregation. Thus, our central focus involves the incorporation of hydrophilic entities, i.e. oligomeric ethylene glycol, into the backbone of an
aromatic polyanhydride to create a potentially suitable protein carrier. The choice of ethylene glycol is motivated by its advantageous properties, including the stealth effect provided to various active macromolecules, its biocompatibility, and its low toxicity. Previous studies have demonstrated that poly(ethylene glycol) is a useful carrier for oligonucleotides and ribozymes when polymerized with an aliphatic polycarbonate. When copolymerized with bulk eroding poly(lactide-co-glycolide) or poly(lactic acid), polyethylene glycol adds the hydrophilicity necessary for a faster degrading system for delivery of peptides and proteins, with the added disadvantage of not preventing potentially deleterious water-protein interactions. This paper focuses on copolymers based on the anhydride monomers 1,6-bis(p-carboxyphenoxy)hexane (CPH) and 1,8-bis(p-carboxyphenoxy)-3,6-dioxaoctane (CPTEG), which contains oligomeric ethylene glycol moieties (the chemical structures are shown in Fig. 1). The synthesis, characterization and erosion mechanism of these novel polyanhydrides are discussed\textsuperscript{5,6}.

![Figure 1. Chemical structures of poly(CPH) (left) and poly(CPTEG) (right). The letters a–i represent the peaks in the $^1$H NMR spectra shown in Fig. 2.](image)

**Materials and Methods**

**Materials**

4-Hydroxybenzoic acid, 1,6-dibromohexane, and triethylene glycol were purchased from Sigma Aldrich (St. Louis, MO); 4-p-fluorobenzonitrile came from Apollo Scientific (Cheshire, UK); acetic anhydride, methylene chloride, potassium carbonate, petroleum ether, toluene, dimethylformamide (DMF), sulfuric acid, acetic acid, and acetonitrile were obtained from Fisher Scientific (Fair Lawn, NJ). Deuterated chemicals for NMR analysis (chloroform and dimethyl sulfoxide (DMSO)) were purchased from Cambridge Isotope Laboratories (Andover, MA).

**Monomer synthesis**

The CPH monomer was synthesized from 4-hydroxybenzoic acid using a procedure that was first developed by Conix\textsuperscript{7}. To synthesize the CPTEG monomer, 45 mL of triethylene glycol, 100 mL of toluene, 300 mL of DMF, and 0.897 mol of potassium carbonate were mixed in a round-bottom flask placed in an oil bath at 170°C. The addition of toluene allowed the azeotropic distillation of water from the reaction mixture prior to reaction. Next, 0.684 mol of 4-p-fluorobenzonitrile was added and allowed to react overnight at 150°C. After cooling, all the solvents were removed using a rotary evaporator. The resulting dinitrile solution was hydrolyzed with a mixture containing equal volumes (50 mL) of water, acetic acid, and sulfuric acid. The reaction was carried out at 160°C under a nitrogen atmosphere. The resulting diacid was precipitated with 1 L of deionized water. A white powder was obtained after successive washes with acetonitrile.

**Polymer synthesis**

Due to problems with prepolymer isolation, poly(CPTEG) and CPTEG:CPH copolymers
were synthesized starting directly from the diacids. In a typical experiment, 2 g of the monomer and 100 mL of acetic anhydride were added to a round-bottom flask and reacted for 30 min at 125°C. The acetic anhydride was removed in the rotary evaporator and the resulting viscous liquid was polymerized in an oil bath at 140°C, under vacuum (<0.03 torr) for 90 min. The polymer was isolated by precipitating from methylene chloride into petroleum ether. The copolymer compositions synthesized were 20:80, 50:50, and 80:20 CPTEG:CPH. Additionally, homopolymers of CPH and CPTEG were synthesized. The CPTEG homopolymer was synthesized by polycondensation of the CPTEG monomer as described above.

**Characterization**

The purity of the monomers and polymers was verified using $^1$H NMR spectra obtained from a Varian VXR-300 MHz NMR spectrometer. Number average molecular weights were estimated by end-group analysis from $^1$H NMR spectra. Perkin Elmer DSC 7 and DMA were used for the thermal characterization. The samples were heated in two cycles from -20 to 110°C at a rate of 5°C/min in the DSC. For the DMA analysis, 100-mg tablets were heated from -20 to 100°C at a rate of 3°C/min, and a three-point bending test was performed at a frequency of 1 Hz, a dynamic force of 90 mN, and a static force of 100 mN. Scanning electron microscopy (SEM) (Hitachi S-2460 N) was used to study the surface and cross-section of the polymer tablets during erosion. The dried tablets were coated with gold prior to imaging.

**Erosion and degradation studies**

Tablets of 100 mg of poly(CPTEG), poly(CPH) and 50:50 CPTEG:CPH copolymer were melt-compressed for 2 min in a Carver press (Wabash, IN) at a pressure of 600 psi and at a temperature just above the melting point of the polymer. Then the tablets were placed into 25 mL of phosphate buffer (0.1M, pH 7.4) in an incubator operating at 37°C and 100 rpm. The buffer was replaced daily. At different time intervals, duplicate samples of tablets were taken out of the buffer for further analysis. The water swelling and the mass loss of the tablets were determined by gravimetric analysis. The surface morphology of the tablets was monitored by SEM.

**CPTEG monomer solubility**

To further characterize the erosion mechanism, CPTEG monomer solubility was determined by dissolving an excess of diacid in 15 mL of phosphate buffer at different pH values. The diacid concentration was calculated from UV absorbance (Shimadzu) using Beer’s law at 288 nm.

**Results and Discussion**

**Structural characterization**

The $^1$H NMR spectra in Fig. 2 confirm the successful synthesis of poly(CPTEG), CPTEG:CPH copolymers, and poly(CPH). The peak designations with respect to deuterated chloroform ($\delta = 7.26$ ppm) was confirmed based on reported values. The aromatic proton peaks of CPTEG and CPH monomers (a-d) have the characteristic chemical shifts in the $\delta = 6.8-8.1$ ppm range. The inner chain protons close to the electronegative oxygen atoms in both monomers (e–h) are represented in the expected range of 3.6–4.4 ppm, and the protected protons in the inner chain of CPH (j, k) are represented at $\delta = 1.7$ and $\delta = 1.5$ ppm. Finally, the acetylated end groups (i) have a chemical shift at $\delta = 2.1$ ppm. The molecular weights of the polymers synthesized ranged from 4,000–14,000 g/mol, as determined by end-group analysis by $^1$H NMR.
Figure 2. $^1$H NMR spectra of (a) poly(CPTEG), (b) 80:20, (c) 50:50, (d) 20:80 CPTEG:CPH copolymers, and (e) poly(CPH).

Thermal characterization

A summary of the thermal properties is shown in Table 1. The glass transition temperatures were determined from the DMA studies. The CPTEG homopolymer and all the CPTEG:CPH copolymers have $T_g$'s below 20°C and hence are rubbery at room temperature. The rubbery state of these polymers at room temperature is desirable for processing into tablets. The DSC studies for the CPTEG homopolymer and the CPTEG:CPH copolymers did not exhibit any melting

Table 1: Thermal characterization

<table>
<thead>
<tr>
<th>Polymer</th>
<th>$T_g$ (°C)</th>
<th>$T_m$ (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Poly(CPTEG)</td>
<td>9</td>
<td>—</td>
</tr>
<tr>
<td>80:20 CPTEG:CPH</td>
<td>7</td>
<td>—</td>
</tr>
<tr>
<td>50:50 CPTEG:CPH</td>
<td>8</td>
<td>—</td>
</tr>
<tr>
<td>20:80 CPTEG:CPH</td>
<td>18</td>
<td>—</td>
</tr>
<tr>
<td>Poly(CPH)</td>
<td>47</td>
<td>143</td>
</tr>
</tbody>
</table>
peaks, indicating that these polymers are amorphous. In contrast, poly(CPH) melts at 143°C and has a $T_g$ of 47°C, indicating that when copolymerized with CPTEG, the crystal formation is disrupted. This is important since polymer crystallinity affects the erosion mechanism of the polymer, since crystalline regions erode more slowly than the amorphous ones. Thus, it is expected that copolymers rich in CPTEG and the poly(CPTEG) homopolymer would have faster erosion rates and erosion mechanisms that deviate from pure surface erosion.

Erosion and degradation

Polymer erosion is a complex process that is determined by numerous factors that include the molecular weight loss (degradation), the swelling, the dissolution and diffusion of oligomers and monomers, and morphological changes\(^5\). The erosion of poly(CPTEG), poly(CPH), and the 50:50 CPTEG:CPH copolymer was monitored by following mass loss, polymer swelling, molecular weight changes, monomer dissolution, and surface morphology.

The mass loss from the polyanhydride tablets was determined by gravimetry (Fig 3). In 28 days the poly(CPTEG) homopolymer lost ~80% of its total mass, which is attributed to the increased hydrophilicity due the oligomeric ethylene glycol in the aromatic polyanhydride. In the same period, less than 5% mass was lost from the highly hydrophobic poly(CPH) homopolymer. The 50:50 CPTEG:CPH copolymer eroded at a rate between that of the two homopolymers. From these results it can be seen that the polymers exhibit distinct erosion profiles that can be controlled by tailoring copolymer composition. These results are also consistent with the DSC studies, which showed that both poly(CPTEG) and the 50:50 CPTEG:CPH copolymers are amorphous, which would lead to faster erosion rates.

![Fractional mass loss from tablets of poly(CPTEG), 50:50 CPTEG:CPH copolymer, and poly(CPH). Error bars indicate standard deviation.](image)

The water swelling in polymers may affect the rate of drug release. In surface-erodible materials, the drug release is mainly controlled by erosion kinetics\(^9\), since no water can penetrate into the system. On the other hand, bulk-erodible polymers release drugs as a result of various processes occurring in unison, including erosion kinetics, swelling, and diffusion. The water
content present in each sample was analyzed (data not shown). Even though the mass loss of poly(CPTEG) and 50:50 CPTEG:CPH copolymer was remarkably different (Fig. 3), the water content of both polymers followed a similar trend. A significant amount of water entered into these tablets, suggesting a transition to a bulk-erodible system as the CPTEG content in the copolymer increased. After 14 days, the water content of poly(CPTEG) exceeded 50%, while in poly(CPH), the water content did not exceed 5%, demonstrating the well-known surface erosion mechanism of these hydrophobic polyanhydrides. The water content in the 50:50 CPTEG:CPH copolymer was in an intermediate range between the two homopolymers for the duration of the erosion studies. The data suggest that the erosion of this family of polyanhydrides (i.e., CPTEG-based) can be tuned from “bulk” to “surface” by copolymerizing with hydrophobic CPH.

The degradation of the polymers was studied using \(^1\)H NMR spectra of eroded tablets in deuterated DMSO (δ ~2.5 ppm) (spectra not shown). As expected, the spectra showed a significant decrease of the peak area of the protons corresponding to the polymer backbone (e–k) and an increase in the end-group acid peak (i) after 28 days of degradation. On day 28, the decreased peaks that still prevailed in the spectra corresponded to the CPH monomer, clearly indicating that CPH is the last monomer released. The molecular weight loss was determined by end-group analysis of \(^1\)H NMR spectra, showing that poly(CPTEG) and the 50:50 CPTEG:CPH copolymer displayed similar rates of molecular weight loss, i.e. 93% for poly(CPTEG) and 85% for the 50:50 CPTEG:CPH copolymer. As expected, the hydrophobic poly(CPH) lost only 8% of its initial molecular weight after 28 days. These studies demonstrate that the added hydrophilicity enhances the degradation rate of the polyanhydride, and this is supported by the water penetration data into polymers containing CPTEG.

The studies indicate that poly(CPTEG) and the 50:50 CPTEG:CPH copolymer have different erosion rates, but similar water swelling and polymer degradation rates. Since erosion is a combination of polymer degradation, water swelling, monomer dissolution, and diffusion, we investigated the solubility of both monomers (Fig. 4). The data indicate that the saturation concentration for CPTEG is at least an order of magnitude greater than that of CPH. This difference in monomer solubility may govern the erosion mechanism of the polymer that can be varied from bulk to surface erosion by increasing the CP content. The solubility data were also used to estimate the logarithmic scales of acidity constants for each diacid, i.e. \(pK_a\). It has been reported that CPH has \(pK_a\)'s at 3.7 and 6.7. Our data shows that CPTEG has \(pK_a\)'s at 5.8 and 8.4, indicating that CPH is a stronger acid. This data is important since the dissolution of the monomers decreases the pH of the microenvironment of an eroding polymer, which in turn limits the monomer solubility, as the dicarboxylic acid monomers become much less soluble at low pH.

Finally, we studied the surface morphology of the tablets during erosion using SEM. The surface morphology of the polymers after 7 and 28 days of exposure to buffer were analyzed (images not shown). The poly(CPTEG) tablets had an homogeneous porosity characteristic of bulk-eroding polymers where water entered easily. In contrast, the 50:50 CPTEG:CPH copolymer exhibited a smooth surface with small erosion fronts throughout the tablet. This behavior is typical of systems that exhibit features of both bulk and surface erosion. The surface-eroding poly(CPH) exhibited a smooth surface throughout the 28-day study, consistent with the water penetration studies discussed previously. These results indicate that by varying the CPTEG content in copolymer systems, the erosion mechanism can be tailored from bulk to surface erosion.
Conclusions

We have synthesized a new class of "bulk-eroding" polyanhydrides with tailored amphiphilicity with potential for controlled drug and protein delivery. When copolymerized with hydrophobic aromatic polyanhydrides, these systems can be tailored from "bulk-eroding" to "surface-eroding". Such a combined erosion mechanism may prevent protein denaturation by avoiding both hydrophobic non-covalent interactions and covalent aggregation of proteins. We are currently evaluating the potential of these novel biomaterials as protein carriers.

References

OXYGEN TOXICITY TO BIFIDOBACTERIA

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ABSTRACT
The enumeration of probiotic microorganisms, especially bifidobacteria, is an important and complex topic because of varied nutritional requirements of bifidobacteria and their strict anaerobic nature. The two factors which are very important in detecting and enumerating bifidobacteria are an adequate culture medium and anaerobic conditions. An experiment was done to see the effectiveness of oxygen reducing membrane fragments (ORMF) on recovery of bifidobacteria and other probiotic microorganisms when added in dilution medium. The addition of ORMF in dilution bottles helped recover more counts of microorganisms in four of the five species studied. Hence, ORMF has the potential for improving the growth of probiotic microorganisms.

INTRODUCTION
The term probiotics came into use at the start of the twentieth century. Metchnikoff (1907) in his article, prolongation of life suggested that man should consume milk fermented with Lactobacillus bulgaricus to prolong life. He believed that specific lactobacilli would displace the harmful microorganisms present in the GI tract. He thought that many of the so-called normal microorganisms present in the GI tract produce “toxins” that could result in the reduction of one’s life span or general well being. Fuller (1989) stated that probiotic is a live microbial feed supplement, which beneficially affects the host animal by improving its intestinal microbial balance. The majority of probiotic microorganisms fall into two genera. They are Lactobacillus and Bifidobacterium, which are inhabitants of human GI tract. The health benefits of probiotic bacteria include controlling growth of undesirable microorganisms, improvement of immune response, improvement in lactose digestion, anticarcinogenic actions, and control of serum cholesterol.

Oxygen Toxicity to Probiotic Microorganisms

Lactobacillus acidophilus and Bifidobacterium spp., commonly used probiotic microorganisms, are human gut-derived organisms and are microaerophilic and strictly anaerobic, respectively. It was observed that the oxygen-scavenging system in probiotic bacteria is either reduced or completely absent. The lack of an electron-transport chain in these bacteria results in the incomplete reduction of oxygen (Talwalkar and Kailaspathy, 2004). Hence exposure to oxygen in these microorganisms causes the accumulation of toxic oxygenic metabolites such as superoxide anion (\(O_2^-\)), hydroxyl radical (\(OH^-\)), and hydrogen peroxide (\(H_2O_2\)) in the cell, eventually leading to cell death. Bifidobacterium
species are considered more vulnerable to oxygen than other probiotic microorganisms. The following Table 1 lists the ways to reduce oxygen toxicity to bifidobacteria.

Table 1: Approaches to Reduce Oxygen Toxicity to Bifidobacteria

<table>
<thead>
<tr>
<th>Approach</th>
</tr>
</thead>
<tbody>
<tr>
<td>Use of Special High-Oxygen Consuming Strains</td>
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<tr>
<td>Oxidative Stress Adaptation</td>
</tr>
<tr>
<td>Use of Ascorbic Acid as an Oxygen Scavenger</td>
</tr>
<tr>
<td>Addition of Cysteine</td>
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<tr>
<td>Packaging Material Impermeable to Oxygen</td>
</tr>
<tr>
<td>Microencapsulation</td>
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<tr>
<td>Oxygen Reducing Membrane Fragments</td>
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</tbody>
</table>

**Oxygen Reducing Membrane Fragments**

Oxygen reducing membrane fragments (ORMF) have been used for quite a while for detection and enumeration of various starter cultures and pathogenic microorganisms. Schnaitman (1970) separated the cell wall of *Escherichia coli* into two fractions. One fraction was enriched with cell wall fragments, whereas the other one was enriched with cytoplasmic membrane fragments. These cytoplasmic membrane fragments obtained from *Escherichia coli* and other cells and tissues remove oxygen converting it to water by an enzymatic process. The removal of oxygen is due to presence of the cytochrome based electron transport system, located in the cytoplasmic membrane of microorganisms.

The first report discussing the use of sterile bacterial membrane fragments and their associated enzymes as reagents for the reduction of dissolved oxygen appeared in 1981 (Adler and Crow, 1981). The membrane fragments rapidly remove all detectable oxygen from an initially saturated complex bacteriological medium. As the enzymes are not consumed or inactivated during this process of removing oxygen, repeated injections of additional oxygen can be tolerated. In order for the membrane-bound electron-transport system to reduce oxygen to water, a suitable organic source of hydrogen must be readily available. Sodium lactate and sodium succinate have been determined to be suitable hydrogen donors for membrane fragments derived from *Escherichia coli* (Adler and Crow, 1981). As the concentration of oxygen dissolved in liquid is always less than 1mM, the concentration of hydrogen donating substrate required for complete and rapid oxygen reduction is correspondingly low. In practice, 1-10 mM concentrations of hydrogen donor are sufficient to produce and maintain complete anaerobiosis (Adler, 1990).

**Application of Oxyrase™ in Food Microbiology and Food Fermentation**

Oxyrase™ is a commercially available ORMF from *Escherichia coli*, obtained from Oxyrase Inc., Mansfield, OH. It creates anaerobiosis in a variety of bacteriological media. The Oxyrase™ has been shown to enhance the recovery of important pathogens such as *Escherichia coli* O157:H7 (Phebus et al., 1993), *Listeria monocytogenes* (Yu and Fung, 1991), *Clostridium perfringens* (Ali et al., 1991), and *Salmonella typhimurium*,...
Oxyrase™ stimulated the growth of various lactic acid bacteria including *Streptococcus thermophilus*, *Lactococcus lactis*, *Lactococcus cremoris*, *Propionibacterium acidipropionici*, *Lactobacillus bulgaricus*, *Lactobacillus acidophilus*, *Lactobacillus plantarum*, *Pediococcus acidilactici* when used at 0.1 unit of Oxyrase™ per ml of broth (Park, 1992). The stimulatory effect was manifested by shorter lag time, faster growth rate, shorter generation time and faster acidity development. Park (1992) also incorporated Oxyrase™ in yogurt formulations (0.1 unit per ml) and studied the yogurt fermentation over a period of five hours. The decrease in pH and increase in acidity was higher with Oxyrase™. The cell population was higher in presence of Oxyrase™ than control.

Oxyrase™ and ORMF obtained from *Escherichia coli* (E-8), and *Gluconobacter oxydans* were added during the fermentation of yogurt, summer sausage, buttermilk, bread, wine, and beer (Tuitemwong, 1993). All membrane fractions significantly stimulated the growth and product formulation of all food products. Addition of membrane fractions reduced the needed production time of these foods (Faster acidity development). Ordonez-Fiallos (1994) applied Oxyrase™ (0.5 unit per ml) in probiotic yogurt formulation and studied fermentation of yogurt over a period of 8 hours. Yogurt cultures, (*Streptococcus thermophilus* and *Lactobacillus bulgaricus*) *Lactobacillus acidophilus*, *Bifidobacterium bifidum* showed higher growth rate and shorter generation time in the presence of Oxyrase™. The greatest effect of Oxyrase™ was observed with *Bifidobacterium bifidum*.

**Application of ORMF for Improving Growth and Recovery of Bifidobacteria**

The ORMF have been successfully applied during formulation of food products as stated earlier (Park, 1992; Tuitemwong, 1993; Ordonez-Fiallos, 1994). Dilution is very important step in enumeration of microorganisms in food products. During the dilution process, mixing of culture by shaking the dilution tube or by vortexing may incorporate oxygen from the head space creating unfavorable condition for growth of bifidobacteria. Collins and Hall (1984) have found improved recovery of bifidobacteria using saline, KH₂PO₄, and Na₂HPO₄ when added in dilution medium. Hartemink and Rombouts (1999) have also observed better recovery of bifidobacteria when compounds such as cysteine HCl, which reduced redox potential, were added to dilution media. The removal of oxygen by the membrane fraction is accompanied by a reduction of approximately 200-300 mV in the redox potential. Hence, there is a great potential for application of ORMF in formulation of food products containing bifidobacteria and in dilution bottles while enumerating bifidobacteria containing food product. The purpose of this work was to investigate the effect of Oxyrase™ in dilution bottles.
MATERIALS AND METHODS

Bacterial Strains

The commercial probiotic finished products, obtained from one probiotic
manufacturing company were analyzed. Each product containing the specific culture,
with their suggested minimum count, was as follows:

Lactobacillus acidophilus Strain A (9.3 log CFU/gram)
Lactobacillus acidophilus Strain B (9.3 log CFU/gram)
Lactobacillus bulgaricus (9.3 log CFU/gram)
Bifidobacterium bifidum (9.3 log CFU/gram)
Bifidobacterium infantis (9.0 log CFU/gram)

All the cultures were obtained from the company in powder form and were kept at
refrigeration temperature till evaluation as suggested by the company. The complete
names of the probiotic organisms and that of the supplier cannot be given for the sake of
confidentiality.

Oxyrase™

Sterile cytoplasmic membrane fragments produced from Escherichia coli were
obtained from Oxyrase Inc., Mansfield, OH. Oxyrase™ was distributed in 50 ml bottles
and kept frozen. Before the experiment, Oxyrase™ was thawed in a refrigerator
overnight.

Media Used for Evaluation

Lactobacillus acidophilus A & Lactobacillus acidophilus B

MRS agar was used for evaluating both the strains of Lactobacillus acidophilus.
MRS broth (Difco, Becton Dickinson and Company, Sparks, MD) and granulated agar
(Difco, Becton Dickinson and Company, Sparks, MD) were added to distilled water. This
mixture was heated, boiled and sterilized for 15 minutes at 250° F at 15 psig pressure.
The pH of the agar was maintained between 6.3 and 6.7

Lactobacillus bulgaricus

MRS-acid agar was used for evaluating Lactobacillus bulgaricus. MRS broth and
granulated agar were added to distilled water. This mixture was heated, boiled and 2.5 ml
of glacial acetic acid was added per liter of agar. This mixture was sterilized for 15
minutes at 250° F at 15 psig pressure. The pH of the agar was maintained between 5.0
and 5.3.

Bifidobacterium bifidum

MRS-Cysteine HCl agar was used for evaluation of Bifidobacterium bifidum.
MRS broth and granulated agar were added to distilled water. This mixture was heated,
boiled, and sterilized for 15 minutes at 250° F at 15 psig pressure. A 10% solution of
cysteine HCl (Sigma-Aldrich, St. Louis, MO) was prepared and was sterilized for 15
minutes at 250°F at 15 psig pressure. One ml of sterilized Cysteine HCl solution was added to 200 ml of MRS agar. The pH of the agar was maintained between 6.3 and 6.7.

**Bifidobacterium infantis**

MRS-Cysteine HCl-Amberex 695 agar was used for evaluation of *Bifidobacterium infantis*. MRS broth and granulated agar were added to distilled water. This mixture was heated, boiled and sterilized for 15 minutes at 250°F at 15 psig pressure. Cysteine HCl solution (10% solution) was prepared and was sterilized for 15 minutes at 250°F at 15 psig pressure. Amberex 695 solution was prepared (20% solution) and was sterilized for 15 minutes at 250°F at 15 psig pressure. 1 ml of sterilized cysteine HCl solution and 10 ml of sterilized Amberex-695 were added to 200 ml of MRS agar. The pH of the agar was maintained between 6.3 and 6.7.

The pour plates were used for all the microorganisms. For bifidobacteria cultures, in addition to pour plates, Modified Columbia Agar (MCA) OxyPlate™ obtained from Oxyrase Inc, Mansfield, OH were also used for evaluation. The important thing about Oxyplate is that it comes in ready-to-use form with Oxyrase enzyme added into the agar. These plates can be incubated aerobically. OxyPlate™ is convenient for enumerating anaerobic organisms since one need not use any anaerobic atmosphere generating systems and hence it simplifies the incubation procedure. Oxyrase Inc suggested that the addition of Oxyrase™ in medium below pH of 6.8 could drastically reduce the count of microorganisms. The media used for enumerating both *Bifidobacterium* spp. (MRS-Cysteine HCl agar and MRS-Cysteine HCl-Amberex 695) had pH less than 6.8. But MCA OxyPlate™ had pH of 7.2 and hence MCA OxyPlate™ might prove helpful for recovery of bifidobacteria.

Flip Top™ Peptone water bottles (FT-PW99) were obtained from Biotrace International Inc, Muncie, IN, USA and were used for dilution. Oxyrase™ obtained from Oxyrase Inc., was added to half of the peptone water dilution bottles at 0.1 unit per ml of peptone water. The other peptone water bottles without Oxyrase™ served as a control.

**Microbial Enumeration**

**Microbial Enumeration of *Lactobacillus acidophilus* A and B, and *Lactobacillus bulgaricus***

Each of the microbial powders was thawed at room temperature for a couple of hours before the experiment. Eleven grams of culture was mixed with 99 ml of peptone water (with no Oxyrase added) in stomacher bags. This mixture was stomached for 2 minutes and was held for 20 minutes. After 20 minutes, it was again stomached for 2 minutes. The culture was then serially diluted in 99 ml of peptone water. One ml and 0.1 ml of appropriate dilutions were plated on blank plates. The respective tempered agar was poured over the culture and it was swirled and mixed. After these plates were solidified, they were incubated anaerobically at 37°C for 72 hours. The anaerobic atmosphere was generated using AnaeroPack™ system (Mitsubishi Gas Chemical America Inc., New York, NY). This system consisted of AnaeroPack system jars and AnaeroPack sachet. This sachet generates carbon dioxide with absorption of oxygen. This system is very
convenient because it does not need any water for generation of carbon dioxide. The anaerobic indicators were used for verifying anaerobic conditions. The whole procedure was repeated for the cultures with addition of Oxyrase™ in dilution bottles. All organisms were plated in duplicates and each experiment was repeated three times.

**Microbial Enumeration of* Bifidobacterium bifidum* and* Bifidobacterium longum**

Each of the microbial powder was thawed at room temperature for couple of hours before the experiment. Eleven grams of culture was mixed with 99 ml of peptone water (with no Oxyrase™ added) in stomacher bags. This mixture was stomached for 2 minutes and was held for 20 minutes. After 20 minutes, it was again stomached for 2 minutes. The culture was then serially diluted in 99 ml of peptone water. One ml and 0.1 ml of appropriate dilutions were plated on blank plates. The respective tempered agar was poured over the culture and it was swirled and mixed. After these plates were solidified, they were incubated anaerobically at 37°C for 72 hours. The anaerobic atmosphere was generated using AnaeroPack™ system. The anaerobic indicators were used for verifying anaerobic conditions. Then 0.1 ml and 0.5 ml of appropriate dilutions were spread plated on MCA OxyPlate™ and were incubated aerobically at 37°C for 72 hours. The whole procedure was repeated for the cultures with addition of Oxyrase™ in dilution bottles. Both the organisms were plated in duplicates and each experiment was repeated three times.

**RESULTS AND DISCUSSION**

Table 2 shows the summary of counts of individual microorganisms with and without Oxyrase™ in dilution bottles. The count shown here is the average of three repetitions. Graph 1 shows the counts of microorganisms in the presence and absence of Oxyrase™. From Table 2 and Graph 1, we can observe that the counts obtained using Oxyrase™ were higher for all microorganisms except *Bifidobacterium bifidum*. This suggests that addition of Oxyrase in dilution bottles helped recover more counts of microorganisms, although the increase in count was not statistically significant (p>0.05).

Oxyrase™ obtained from cytoplasmic membrane fragments contains enzyme systems that catalyze the reduction of dissolved oxygen to water. Until now Oxyrase™ has been shown to be effective for various facultative anaerobic microorganisms. In this experiment, 0.1 unit of Oxyrase™ per ml of diluent was employed. The concentration of enzyme used in this experiment (0.1 unit/ml) might not be sufficient for bifidobacteria as it is a strict anaerobe and cannot tolerate small amounts of oxygen.

Niroomand (1993) studied the effect of different concentrations of Oxyrase™ (0, 0.1, 0.2, 0.4, 0.6, and 0.8 unit/ml) on growth of *Campylobacter jejuni* and *Campylobacter coli* in brucella enrichment broth at 42°C in shaking and non-shaking water baths. The generation times of most strains were inversely related to the concentration of Oxyrase™ added. The author has reported that for most *Campylobacter* strain the shortest generation time (faster growth) occurred in brucella broth with ≥ 0.6 unit/ml. Hence this author
suggested that Campylobacter, being highly sensitive to oxygen, a higher concentration of Oxyrase™ was needed to achieve the maximum growth of this organism.

Yu (1991) studied the effect of different concentrations of Oxyrase™ in Frazer broth on growth of Listeria monocytogenes strains and found that growth was directly related to the concentration of Oxyrase™. Park (1992) studied the growth of Streptococcus thermophilus and Lactobacillus bulgaricus in different quantities of Oxyrase™ (0, 0.03, 0.1, and 0.3 unit/ml). The author has also noted that the growth of these starter cultures was related to the concentration of Oxyrase™. The growth rate was increased as concentration of Oxyrase™ was increased. This suggests that the present experiment could be repeated by employing higher concentrations of Oxyrase™ for improving the growth of bifidobacteria.

MCA Oxyplates obtained from Oxyrase Inc were used for both the strains of bifidobacteria. These plates contain Oxyrase™ and hence can be incubated aerobically. The OxyPlate™ did not prove to be effective in this experiment. The plates could not recover microorganisms properly. They could not produce any colonies in some repetitions and for some repetitions although they produced colonies, they were too small to enumerate. This suggests that MCA OxyPlate™ was not helpful for enumerating these strains of microorganisms. The possible reason could be that MCA agar may not be a good choice for strains of bifidobacteria used in this experiment. As stated previously, Bifidobacterium spp. vary widely in their nutritional requirements for growth; hence no one selective medium is appropriate for all species.

Hence this experiment could be repeated using higher Oxyrase™ concentration. Also one can validate the effectiveness of OxyPlate™ by selecting some different media than MCA.

CONCLUSION

It was possible to recover as many microorganisms as anticipated based on the information supplied by the company. The presence of Oxyrase™ in dilution bottles proved to be helpful giving comparable or somewhat higher counts compared to the absence of Oxyrase™. The increase in counts of microorganisms was not statistically significant. In all cases, the mean values were larger than values that were expected based on the information supplied by the company. It appears that the loss of viable cells due to the dilution process was modest even when Oxyrase™ was not used. MCA OxyPlate™ employed in the experiment did not recover bifidobacteria. The experiment could be repeated using higher concentrations of Oxyrase™ and selecting media other than MCA for Oxyplate.

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**ACKNOWLEDGEMENT**

Financial support from Kansas State University for Amit Apte provided partial support for this research.
1. *Lactobacillus acidophilus* I
2. *Lactobacillus acidophilus* II
3. *Lactobacillus bulgaricus*
4. *Bifidobacterium bifidum*
5. *Bifidobacterium infantis*

**Figure 1:** Effect of Oxyrase™ on recovery of probiotic microorganisms

**Table 2: Effect of Oxyrase™ on measured values of colony forming units of cells**

<table>
<thead>
<tr>
<th>Name of Organism</th>
<th>No Oxyrase™ in dilution medium</th>
<th>Oxyrase™ in dilution medium</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Lactobacillus acidophilus</em> I</td>
<td>$9.65 \pm 0.08^a$</td>
<td>$9.73 \pm 0.10^a$</td>
</tr>
<tr>
<td><em>Lactobacillus acidophilus</em> II</td>
<td>$9.32 \pm 0.07^a$</td>
<td>$9.57 \pm 0.12^a$</td>
</tr>
<tr>
<td><em>Lactobacillus bulgaricus</em></td>
<td>$9.58 \pm 0.21^a$</td>
<td>$9.60 \pm 0.16^a$</td>
</tr>
<tr>
<td><em>Bifidobacterium bifidum</em></td>
<td>$9.64 \pm 0.05^a$</td>
<td>$9.50 \pm 0.12^a$</td>
</tr>
<tr>
<td><em>Bifidobacterium infantis</em></td>
<td>$9.15 \pm 0.22^a$</td>
<td>$9.25 \pm 0.25^a$</td>
</tr>
</tbody>
</table>

$^a$Each value in the table is mean ± standard deviation of three trials. Means (within a row) with the same letter are not different (p>0.05)
Targeting L-Methioninase to Human Cancer Cells

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Abstract

Tumor cells have an elevated requirement for methionine, an essential amino acid. In contrast, normal cells are relatively resistant to exogenous methionine restriction and grow well when methionine is substituted with homocystine. Methionine depletion in vicinity of tumor cells by methioninase, an enzyme which specifically degrades methionine, inhibits tumor growth. The present study developed a novel fusion protein targeted specifically to the urokinase receptor on tumor cells. The fusion protein contains two components: the amino terminal fragment (ATF) of human urokinase (amino acids 1-49) and L-methioninase (containing 398 amino acids), an enzyme that catalyzes the breakdown of methionine to $\alpha$-ketobutyrate, methanethiol, and ammonia. The urokinase receptor is overexpressed on many different types of cancer cells.

The ATF fragment (1-49 amino acids) gene and the L-methioninase gene were fused and ligated to pET 30 Ek/LIC expression plasmid and transformed in \textit{E. coli} strain BL21(DE3) cells. Fusion protein was expressed along with the histidine (His)$_6$ tag, and it was purified by immobilized metal affinity chromatography. The (His)$_6$ tag was cleaved by using HRV 3C protease. Binding of fusion protein to urokinase receptors on surface of MCF-7 breast cancer cells was demonstrated by measuring the displacement of the fusion protein from MCF-7 human breast cancer cells by urokinase. Treatment of MCF-7 breast cancer cells and SK-LU-1 lung cancer cells with fusion protein over the concentration range of $10^{-8}$M to $10^{-6}$M showed an effective dose-dependent inhibition of both migration and proliferation index in culture wounding assays. \textit{In vivo} studies in nude mice, the fusion protein significantly reduced the tumor size and prevented metastasis of MCF-7 human breast tumors. In conclusion, this fusion protein appears to have potential as a therapeutic agent for cancer treatment.

Introduction

Methionine dependence, the elevated methionine requirement for tumor cell proliferation, is the property of the majority of tumor cell types tested. Since the beginning of the 1970s, studies have revealed that numerous human tumor cells are characterized by their inability to proliferate when methionine is replaced by its immediate precursor homocystine, whereas normal cells grow in such a medium [1, 2].
Methionine-dependent cells arrest in the G2 and G1 phases of the cell cycle and subsequently die at methionine concentrations less than 5 μM regardless of high concentrations of homocystine precursors and folates [3]. Reduction of plasma methionine can be achieved with the use of the enzyme L-methionine-α-deamino-γ-mercaptopmethanelyase, also known as L-methioninase. This enzyme degrades specifically methionine to α-ketobutyrate, methanethiol and ammonia. A therapeutic approach to enzymatic methionine depletion was first reported in 1973 [4]. There have been several therapeutic strategies to target the methionine dependence of the cancer cells.

The progression of cancer cell invasion and metastasis is centered on the ability of tumor cells to produce and recruit proteolytic enzymes. Among the diverse proteolytic enzyme systems produced by human cancers, the plasminogen activator-plasmin system is involved in cancer cell invasion and metastasis. Urokinase-plasminogen activator (uPA) enzyme, part of plasminogen activator-plasmin system, is the key initiator of the extracellular proteolytic cascade driving cellular invasiveness [5-7]. Urokinase secreted by tumor cells exists as free enzyme or bound to the cell surface receptor, uPAR. Binding to uPAR significantly increases the rate of cell surface-associated plasminogen activation by urokinase and can serve to spatially focus its activity. uPA in particular is implicated in various malignancies including cancer of breast, lung, bladder, stomach, cervix, kidney, etc. [5-7]. Higher levels of urokinase have been correlated with poor patient prognosis and also implicated in extracellular matrix degradation, tumor cell invasion, angiogenesis and metastasis. Studies have shown that residues 12-32 are critical for binding to the receptor [8].

In order to specifically target L-methioninase to breast cancer cells, we have combined L-methioninase in a fusion protein that contained the amino terminal fragment of urokinase, which binds to the urokinase receptor expressed on the surface of the cancer cells. Our major hypothesis is that this fusion protein would bind specifically to urokinase receptors and produce selective cytotoxicity to methionine-dependent breast and lung cancer cells.

Materials and methods

Materials: Synthetic oligonucleotides were produced by the Molecular Biology Resource Facility at the University of Oklahoma Health Sciences Center. Vector pET-30 Ek/LIC, HRV 3C protease, NovaBlue and BL21(DE3) E. coli cells were obtained from Novagen (Madison, WI).

Construction of recombinant expression plasmids: The expression vectors pET-30/Ek/LIC/ATF-Meth and pET-30/Ek/LIC/Meth were constructed as follows: The DNA sequences encoding the ATF-methioninase fusion protein and L-methioninase were amplified from pKK223-3/ATF-Meth [9] by the polymerase chain reaction using the Expand High Fidelity PCR system (Boehringer Mannheim, Indianapolis, IN). The sequence of ATF-methioninase is as follows: N-(amino acids 1-49 of human urokinase A chain)-Gly-Ser-Gly-Ser-Gly-(L-methioninase from P. putida). Forward and
reverse primers used for PCR for ATF-methioninase and L-methioninase were as follows:

(a) the primer ATF 5' (GACGACGACAAGA7GCTTGAAGTCTCCTTTCAGGG ACCCAGCAATGAACCTCATTCAAGTTCC) introduced at the 5' end of the ATF-methioninase DNA sequence an LIC cloning site (italics) and an HRV 3C protease site (underlined);

(b) the primer METH 3' (GAGGAGAAAGCCCGGTATCATGCACACCGCCTCC AATGCCCAACTCG) introduced at the 3' end of the ATF-methioninase or L-methioninase DNA sequence an LIC cloning site (italics);

(3) the primer METH 5' (GACGACGACAAGA7GCTTGAAGTCTCCTTTCA GGGACCCCGCGACTCCATAAACCCCGC) introduced at the 5' end of the L-methioninase DNA sequence an LIC cloning site (italics) and an HRV 3C protease site (underlined).

PCR gene fragments were agarose gel-purified prior to ligation according to the Qiagen protocol (BIO101, Vista, CA). The PCR product was annealed to the pET-30 EK/LIC linear vector and transformed into NovaBlue cells according to the Novagen protocol. This construction results in an N-terminal His-tag sequence with an integrated thrombin cleavage site, enterokinase cleavage site, and an engineered HRV 3C protease cleavage site next to the start of ATF or L-methioninase. (HRV 3C protease cleaves the sequence LEVLFQ~GP). DNA sequences were verified by sequencing at the Oklahoma Medical Research Foundation (Oklahoma City).

**Site-directed mutagenesis of ATF-methioninase:** The plasmid pET30 EK/LIC/ATF-Meth was used as a template for site-directed mutagenesis using PCR. The forward and reverse primers that are complementary to each other create a mutation at the 114th residue of L-methioninase (Y114F) are as follows:

(a) the primer mMETH 5' (GCGCACCTTGTTTGGCTGCACCTTTG);

(b) the primer mMETH 3' (CAAAGGTGCAGCCAAACAAGGTGCGC).

PCR was performed according to Stratagene protocol (La Jolla, CA). Colonies with the mutation were identified by DNA sequencing, and plasmid from these colonies was purified and then transformed into BL21(DE3) cells.

**Expression and purification of recombinant proteins:** E. coli host strain BL21(DE3) harboring ATF-methioninase, mutated ATF-methioninase, or L-methioninase was grown in 50 ml of LB medium containing 35 μg/ml kanamycin overnight at 37 °C with shaking. This cell culture was added to 1 liter of fresh culture medium, and the culture was grown with shaking at 37 °C. When the absorbance at 600 nm reached 0.5, recombinant protein expression was induced by adding isopropyl β-D-thiogalactopyranoside (IPTG) to 0.4 mM concentration, and shaking was continued at 30 °C for 5 h. The cell pellets were collected by centrifugation and were resuspended in 40 ml of sonication buffer (0.05 mM N-p-tosyl-L-phenylalanine chloromethyl ketone (TPCK), 1 mM phenylmethysulfonyl fluoride (PMSF), 1% ethanol, 0.02 mM pyridoxal phosphate, 0.01% β-mercaptoethanol, 0.02 M sodium phosphate, pH 7.4). The suspended cells were sonicated at 4 °C for a total of 2.5 min at 4.5 W/ml (550 Sonic Dismembrator, Fisher Scientific, Pittsburgh,
The lysate obtained was centrifuged at 12,000 \( g \) for 30 min to remove the cell debris. Subsequent steps were carried out at 4 °C.

After adding imidazole (40 mM) and NaCl (500 mM) to the lysate, it was fed to a 5 ml HisTrap chromatography column (Amersham Biotech, Piscataway, N.J.) equilibrated with wash buffer (20 mM sodium phosphate, 40 mM imidazole, 500 mM NaCl, 0.02 mM pyridoxal phosphate, pH 7.4). The column was washed with the wash buffer, and then His-tagged recombinant protein was eluted by elution buffer (20 mM sodium phosphate, 500 mM imidazole, 500 mM NaCl, 0.02 mM pyridoxal phosphate, pH 7.4). Eluted protein was dialyzed against 20 mM sodium phosphate buffer at pH 7.4 containing 0.02 mM pyridoxal phosphate. The cleavage of N-terminal His-tag was achieved by use of HRV 3C protease (0.5 U/mg) with the recommended buffer added (1.5 M NaCl, 0.5 M Tris-HCl, pH 7.5) for 8 h at 4 °C. Cleaved protein was fed again onto a 5 ml HisTrap column, and pure protein was eluted in a linear gradient of 0-0.5 M imidazole. Purified protein was dialyzed against 20 mM sodium phosphate buffer at pH 7.4 containing 0.02 mM pyridoxal phosphate and 0.1 M NaCl, and this formulation was flash frozen using liquid nitrogen and then lyophilized in tubes at a concentration of 1-2 mg/ml.

The enzymatic activity of L-methioninase was measured using L-methionine as a substrate by the spectrophotometric determination of \( \alpha \)-ketobutyrate with 3-methyl-2-benzothiazolone hydrazone hydrochloride [10]. Total protein was determined using the Bradford assay with bovine serum albumin as a standard (Bio-Rad, Hercules, CA). Samples were analyzed by denaturing gel electrophoresis using the SDS-PAGE method with staining by Coomassie blue [11]. Amino-terminal protein sequencing was performed by the Molecular Biology Resource Facility at the University of Oklahoma Health Sciences Center.

Cell culture: MCF-7 human breast cancer cells were provided by the Michigan Cancer Foundation. The cells were maintained as monolayer cultures in RPMI 1640 medium (without phenol red) supplemented with 2 mM \( L \)-glutamine, gentamicin (50 \( \mu \)g/ml), penicillin (100 U/ml), streptomycin (100 \( \mu \)g/ml), estradiol (10\(^{-11} \)M) (all from Sigma, St. Louis, MO) and 5% bovine calf serum (HyClone, Logan, UT) as previously described [12]. In some experiments, the culture medium was methionine-deficient or the methionine was replaced with homocystine. The medium was filter-sterilized and stored at 4°C prior to use.

Cell binding assay and culture wounding assay: These assays have been described previously [13]. These experiments are done using mutated ATF-methioninase, L-methioninase, and buffer (20 mM sodium phosphate buffer at pH 7.4 plus 0.02 mM pyridoxal phosphate) as control.

Mouse xenograft model: In this study, MCF-7 human breast cancer cells (5 x 10\(^5\)), suspended in Matrigel were injected into the flank of nude mice. These cells are stably transfected with the \( \beta \)-galactosidase reporter gene so that tumor metastasis could be determined and quantified. The longest perpendicular diameters of tumors were measured...
twice weekly to document tumor growth. Tumor measurements were converted to tumor volume \((V)\) using the formula: \(V = \frac{W^2 \times Y}{2}\); where \(W\) and \(Y\) are the smaller and larger perpendicular diameters, respectively. The body weight of mice was also measured twice weekly. The development of tumor masses was monitored over a period of 30 days. The animals were then randomly placed into treatment groups. Treatment groups received either the fusion protein (three mice each treated with 12 \(\mu\)l/day at 5 \(\times\) 10^{-6} M, equal to 12 \(\mu\)g/day assuming a molecular weight of 196,000 Da for the homotetrameric fusion protein) or vehicle in the control group (two mice) administered by continuous infusion s.c. to the tumor site over a period of 14 days using an Alzet osmotic infusion pump.

**Data analysis:** Multiple group comparisons were conducted using ANOVA and Student's \(t\)-test for pair-wise comparisons. Group differences resulting in \(P\) values of less than 0.05 were considered to be statistically significant.

**Results**

*Expression and purification of ATF-methioninase, mutated ATF-methioninase, and L-methioninase:* All the proteins were expressed in soluble form 30 °C in *E. coli* BL21(DE3) cells after transformation with the recombinant plasmid. These proteins were found to be insoluble when they expressed at 37 °C. The SDS-PAGE results in Figure 1 show the overexpression at 30 °C of the ATF-methioninase fusion protein in a clone containing the recombinant plasmid and the increasing purity of the fusion protein as the purification progressed. Complete cleavage at the HRV 3C protease site to remove the His-tag was obtained using HRV 3C protease (Figure 1, lanes 3 and 4). The purity of the fusion protein from the final chromatography was estimated to be 94% using Quantity One densitometry software (Bio-Rad, Hercules, CA). Similar expression and purity was also obtained with mutated ATF-methioninase and L-methioninase (results not shown). Amino acid sequencing of the purified ATF-methioninase fusion protein was performed on the first eight amino-terminal amino acids. The sequence was identical to the amino-terminus of the urokinase A chain (Ser-Asn-Glu-Leu-His-Gln-Val-Pro) for 90% of the protein.

After lyophilization and the addition of water to reconstitute the purified protein sample to its volume before drying, the specific L-methioninase activity for pure ATF-methioninase, mutated ATF-methioninase, and L-methioninase was determined to be 13.9 U/mg total protein (average for four separate runs of expression of expression and purification), 0 U/mg total protein (average for two runs), and 14.6 U/mg of total protein (average for two runs), respectively. The loss of activity of ATF-methioninase and L-methioninase during the process of lyophilization and reconstitution with water was small (6-8%). When NaCl was not added to the protein solution before lyophilization, there was a greater than 90% loss of enzyme activity.
Figure 1. SDS-PAGE analysis with Coomassie blue staining of the expression and purification of ATF-methioninase fusion protein (position indicated by the arrow). The fusion protein was expressed from plasmid pET-30/Ek/LIC/ATF-Meth in E. coli BL21(DE3) cells at 30 °C (lane 1 whole cells, lane 2 soluble lysate, lane 3 eluted fraction from first metal affinity chromatography, lane 4 eluted fraction after cleavage with HRV 3C protease, lane 5 pooled fractions from second metal affinity chromatography, M marker proteins with molecular masses indicated on the left in kiloDaltons).

**Specific binding of ATF-methioninase to MCF-7 cells:** In these experiments, the displacement of the fusion protein from urokinase receptors on MCF-7 cells was determined. As shown in Fig. 2, increasing concentrations of urokinase over the concentration range $3 \times 10^{-10}$ to $3 \times 10^{-6}$ M produced a dose-related displacement of fusion protein. The urokinase concentration necessary to produce a 50% displacement of fusion protein was determined to be approximately $10^{-8}$ M. It was also determined that human epidermal growth factor (EGF), over the same concentration range, did not produce any significant displacement of the fusion protein.
Figure 2. Urokinase-induced displacement of fusion protein from membrane binding sites in MCF-7 cells. The data presented are summarized from two experiments. The dotted line indicates the concentration of human urokinase that produced a 50% displacement of fusion protein.

**Inhibitory effects of ATF-methioninase:** The effects of the fusion protein were examined over the concentration range $10^{-6}$ to $10^{-8}$ M on MCF-7 breast cancer cells as shown in Figs. 3 and 4 and on SK-LU-1 lung cancer cells as shown in Figs. 5 and 6. In these experiments, the fusion protein produced a dose-related inhibition of both the proliferation and migration index of MCF-7 cells on days 2 and 3 following fusion protein treatment ($p < 0.05$). Treatment of MCF-7 and SK-LU-1 cells with L-methioninase produced a much smaller reduction of proliferation and migration, which was significant ($p < 0.05$) for SK-LU-1 cells only at the highest protein concentration and on day 2.
Figure 3. Dose-response effect of the fusion protein on MCF-7 cell proliferation index. Each bar represents the distance (mean±SEM from 10 to 12 microscopic fields) of cell migration into the wounded area. Block arrow indicates the most effective dose (10⁻⁶ M) of fusion protein on cancer cells.

Figure 4. Dose-response effect of fusion protein on MCF-7 cell migration index. Each bar represents the number of cells in the wounded area (mean±SEM from 10 to 12 microscopic fields). Block arrow indicates the most effective dose (10⁻⁶ M) of fusion protein on cancer cells.
Figure 5. Dose-response effect of fusion protein on SK-LU-1 cell proliferation index. Each bar represents the number of cells in the wounded area (mean±SEM from 10 to 12 microscopic fields). Block arrow indicates the most effective dose ($10^{-6}$ M) of fusion protein on cancer cells.

Mouse Xenograft Model: The results of this study as shown in Fig. 7 demonstrated that the fusion protein was not cytotoxic to the nude mice since none of the treated animals died or showed signs of whole animal or organ cytotoxicity during the 14-day treatment period. The total number of cancer cells/gram of tissue was reduced by an average of 50% in the fusion protein treatment group compared to the control group. Further, lung
metastases were found in all of the control animals, while none were found in the fusion protein treated mice.

![Cell # Per Gram Tissue Data](image.png)

Figure 7. Total number of cancer cells per weight of tissue after 2-week treatment of nude mice with ATF-methioninase fusion protein or vehicle control. Mice were injected with MCF-7 human breast cancer cells 30 days before treatment started.

**Discussion**

We constructed the fusion protein ATF-methioninase by doing PCR ligation of gene encoding ATF-methioninase, mutated ATF-methioninase, and L-methioninase using pKK223-3/ATF-Meth [9] as template. These fusion proteins were produced in pure form from *E. coli* and purified to near homogeneity with two chromatography steps. The ATF-methioninase fusion protein had methioninase activity and bound to urokinase receptor on MCF-7 breast cancer cells. Full length fusion proteins were synthesized when
compared to the cleavage of fusion protein purified protein in previous described methods[13]

The full length chain of urokinase is required for it to be catalytically active, and this process involves the internalization of urokinase when it is bound to uPAR. Since the fusion protein comprises of only 49 amino acids of urokinase, it would make the fusion protein bind to the receptor without getting internalized. The relative affinity of ATF-methioninase for the cells (50% competition at $10^{-8} M$, Fig. 2) is 10-fold lower than that reported by others for the displacement of a larger ATF (1-135) by urokinase from cultured cells [14], which consistent with reduced binding strength for other urokinase ATF's that are less than 135 amino acids in size [8]. The ATF-methioninase fusion protein inhibited the migration and proliferation index of MCF-7 cells over the concentration range $10^{-5}$ to $10^{-8} M$ in a dose-dependent manner over a period of 3 days, and this fusion protein was effective in inhibiting the cancer metastasis in vivo in nude mice. Work is in progress to produce a fusion protein containing ATF1-135 and L-methioninase and test its binding and cytotoxicity to cancer cells.

In recent studies, methods such as polyethylene glycol conjugation (PEGylation) of the methioninase enzyme have reduced the toxicity observed, provided a longer half-life of L-methioninase depletion, and have less associated immunological reactivity [15-17]. Addition of PEG protects the surface of the protein by increasing the molecular size, thereby increasing the circulating time of the enzyme by reducing its renal clearance and degradation by proteolytic enzymes. Future studies will evaluate the PEGylation of ATF-methioninase.

References


Exploration of Family 1 Carbohydrate Binding Module Structure and Function by Automated Docking

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Abstract
The carbohydrate binding modules (CBMs) of all fungal cellulases possessing them are classified by amino acid sequence similarity into CBM family 1. The only three-dimensional structure available for this family has no ligand associated with it. For this reason it has not been possible to clearly understand the mechanism of action of this domain, although it has been hypothesized that it binds to a cellulose fibril surface while detaching one cellulose chain from it. We have used AutoDock to bind cellooligosaccharide chains to the flat face of the CBM presumably adjacent to the fibril and to its much rougher opposite face. Only one chain binds to each side, in both cases in only one conformation. We have computed binding energies and forces for cellooligosaccharides of different lengths, showing the processive action of the domain in moving along the fibril surface and detaching a single chain from it. The CBM exerts a force on cellooligosaccharide chains of different lengths that is nearly always positive in the direction of burrowing into the fibril. When the CBM is attached by a highly glycosylated linker of ~35 residues to cellbiohydrolases I or II, each of which has its active site located in a tunnel, the detached chain then passes through their tunnels to be cleaved into cellobiose molecules. With endoglucanases having active-site clefts, the chain is cleaved into fragments of different lengths. This use of computation to more clearly elucidate the mechanism of this important CBM demonstrates its great utility in supplementing and extending experimental observations.

Introduction
The *H. jecorina* Cel7A cellulose binding domain (CBD) belongs to carbohydrate-binding module Family 1 (CBM1), whose members are almost exclusively fungal. Three-dimensional NMR of this domain reveals a wedge, one face being flat and hydrophilic, with conserved aromatic residues stacking on the crystalline cellulose surface. The other face is rougher and less hydrophilic.

We have used AutoDock (Scripps Research Institute, La Jolla, CA) to explain how the two domains collaborate to separate cellulose chains from the crystal and then to cleave them. AutoDock is a small-molecule docking program that searches the ligand conformational space for the conformer with the lowest sum of its internal energy plus its interaction energy with the enzyme, and we have used it to supplement knowledge of the structure and function of glucoamylase, β-amyrase, α-1,2-mannosidase, surfactant protein D, phospholipase D, Cel7A, and Cel7B. In this work, docking energies of and forces on cellooligosaccharides docked to the *H. jecorina* Cel 7A CD and CBD were computed to help explain its processive action and ability to disrupt crystalline cellulose.

CBD Docking
The CBD flat face in *H. jecorina* Cel7A is formed by three tyrosine residues, Y466, Y492, and Y493, making a carbohydrate-binding motif. Mutagenesis studies point to their involvement in binding cellulose and soluble Cel6. It is not clear, however, if the rough face is involved in the binding. A P477R mutation there in intact Cel7A caused significant reduction in
enzyme binding and activity toward crystalline cellulose.Mutation of the same residue on isolated CBD did not significantly affect its binding to cellulose, and it was postulated that the rough face is not likely involved in this. Our docking studies, however, point to involvement of both faces, with binding energies of Cel4, Cel5, and Cel6 being significant and equal on each face (Table 1). Binding to either CBD face is weaker than to the CD, the average binding energy per glucosyl residue for the CBD being less negative by ~20 kcal/mol. Forces on the CBD are in the direction of detaching the cellulose chain (Table 1).

Table 1. Energies and forces exerted on substrates by the CD

<table>
<thead>
<tr>
<th>Docked substrate</th>
<th>Docked energy (kcal/mol)</th>
<th>Net force (pN)</th>
<th>Net force along the cellulose chain (pN)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CBD - flat face</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cel4</td>
<td>-148.05</td>
<td>294</td>
<td>183</td>
</tr>
<tr>
<td>Cel5</td>
<td>-180.95</td>
<td>296</td>
<td>156</td>
</tr>
<tr>
<td>Cel6</td>
<td>-184.31</td>
<td>340</td>
<td>170</td>
</tr>
<tr>
<td>CBD - rough face</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cel4</td>
<td>-144.11</td>
<td>111</td>
<td>45</td>
</tr>
<tr>
<td>Cel5</td>
<td>-175.50</td>
<td>93</td>
<td>-14</td>
</tr>
<tr>
<td>Cel6</td>
<td>-184.27</td>
<td>178</td>
<td>74</td>
</tr>
</tbody>
</table>

The discrepancy observed in the effect of the P477R mutation between the intact enzyme and isolated CBD may be because CBD burrowing is necessary for chain binding to the rough face. Atomic-force micrographs of bound Cel7A on crystalline cotton fiber surfaces show evidence of this. It is also possible that binding to the rough face may occur in synergism with chain-end binding to the CD tunnel, and therefore binding could be significantly affected for the P477R mutation in intact Cel7A.

Different cellulose families have evolved a similar CBD design despite differences in size and topology. A once irreversibly bound Cellulomonas fimi CBD can diffuse on the crystalline cellulose surface. Also, the CBD can independently aid the non-hydrolytic disruption of cellulose crystal surfaces. Interestingly, the direction of the net force of CBD-bound cellobiose is along the plane perpendicular to the cellulose surface and along the cellulose chain axis (Fig. 1). From Newton’s Third Law, an equal and opposite force should act on the CBD. This force would be directed in the intended direction of CBD motion for processivity, i.e. towards the cellulose-chain nonreducing end, and this probably biases diffusion in this direction. The CBD, therefore, seems to be designed as a Brownian ratchet.

Binding to the rough face (Table 1, Fig. 1) may be necessary to correctly orient the cellulose chain to enter the CD tunnel and to use the force generated by the CD on the substrate to perform the mechanical work of stripping cellulose chains from the crystal surface. Since this involves hydrogen-bond breakage, the direction in which the force is applied will greatly affect the efficiency of the work performed. The wedge, by lifting cellulose chains from the crystal surface, bends interchain hydrogen bonds, therefore making them easier to break.

In spite of similar overall function, CBDs even within the same family may differ in affinities
or binding-site preferences. Since the flat faces are conserved within CBM1 while the rough faces are not, these differences may be due to the properties of the rough faces.

Two mechanisms can be envisaged for binding intact Cel7A to crystalline cellulose: via the flat face, followed by unidirectionally-biased diffusion until the CD encounters and binds a free chain end, or by synergistic binding to a free chain end by the CD and the rough face.

![Figure 1. Binding of cellohexaose to the flat (lower) and rough faces of CBM1 domain, showing net forces (in pN) exerted by the protein on each ligand.](image)

**Linker**

The CBD and CD of *H. jecorina* Cel7A are separated by a highly O-glycosylated, somewhat flexible linker of ~36 amino acids, resulting in a extended enzyme conformation. Linker length also affects enzymatic activity. Since cellulose chains are linear and rigid due to intra-molecular hydrogen bonding, the distance between the two domains may be important for correct chain-end orientation at the CD tunnel mouth. Also, the linker must be sufficiently stiff to transmit the force generated by the CD through the CBD to the crystalline cellulose surface, so that the cellulose chain end is pulled away from the surface.

**Concerted Action of the CD and CBD**

Once the enzyme is secreted into the extracellular medium, either the CD or the CBD first binds crystalline cellulose. Binding by the CBD would require it to diffuse on the surface until it finds a free cellulose chain end, which then fills the CD tunnel. Since Cel9TS produces a 501 pN force on the substrate (Table 1), greater than the maximal hydrogen-bond bending force of 250 pN, this is probably the stage where the chain is ripped off the surface. After the reaction occurs and Cel2 is expelled from the CD tunnel, the force on Cel2 propels the CD on the cellulose chain, thus starting the next catalytic cycle. Meanwhile, the CBD wedges itself against the newly-formed gap between the cellulose chain end and the crystal surface. Hypothetical binding of the two domains to surface cellulose chains is shown in Fig. 2.
Figure 2. Hypothetical concerted action by CD (above, with transparent tunnel) and CBM (below) to detach cellulose chain from fibril and to cleave cellobiose units from its reducing end by the CD. Linker is attached to CD and CBM at dark parts of both structures. It is not shown because its structure is unavailable.

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References


Environmental Knowledge and Assessment Tool

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Abstract

Designed and built by the team of M2 Technologies, Kansas State University, and CABEM Technologies as members of the National Environmental Evaluation and Remediation Consortium (NEER), the Environmental Knowledge and Assessment Tool (EKAT) is a Web-based research, project management, decision-support tool to identify, research, and evaluate environmental and pollution prevention options, and safety-related issues for products and systems. Originally a concept of the Marine Corps System Command (MARCORSYSCOM) in order to better integrate their environmental requirements into their systems acquisition program, the tool’s design also makes it useful to other federal and state agencies, communities, industry and small businesses.

EKAT presents basic information on technical and regulatory requirements and 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 use. One of the most beneficial aspects of EKAT is its ability to serve as a preliminary environmental screening tool for regulatory requirements and potential issues of concern. EKAT gives the user the ability to quickly screen chemicals against several lists of federally regulated chemicals and against lists of chemicals with known toxicity. Screening against lists of state-regulated chemicals is also possible for a limited number of states. The user may estimate emissions from equipment or processes using the EKAT emissions calculator, which relies on EPA approved emission facors. A NEPA (National Environmental Policy Act) decision tree allows the user to consider and document NEPA-related decisions. Information resource pages and guidance documents in EKAT provide the user with additional environmental information in an easy to read format.

Introduction

It is commonly accepted that the latter half of the 20\textsuperscript{th} Century ushered in what has been come to be known as the ‘Information Revolution.’ In some ways the advent of the Internet and World Wide Web has made accessing and sharing information and ideas much more efficient; and to some extent, it has made it less so. While there is an ample
amount of quality information available, one must be willing to sift through much that may not be truly pertinent to the topic to get to those ‘nuggets of information’ that are desired. The same holds true for someone looking for information on a variety of environmental issues and regulatory compliance.

Many organizations, associations, or agencies have tried to assist their members or clients by providing a central repository of information that has been deemed valuable and credible. Such endeavors have created database and software resources for the agricultural, chemical, legal, and medical professions, just to name a few. One such example is the FAO Statistical Databases, an on-line database maintained by the Food and Agricultural Organization of the United Nations. The United States (U.S.) Environmental Protection Agency and the U.S. Fish and Wildlife Services, along with other federal agencies, have done an excellent job of making environmental information available to the public.

One of the goals, when developing the Environmental Knowledge and Assessment Tool (EKAT), was to find a way to organize the multitudes of environmental information maintained by federal agencies and other organizations, so that those who may not have educational or professional background experiences in said areas can find relevant information quickly and easily. Tools in EKAT are also developed for use by those who are not necessarily environmental professionals.

EKAT is a Web-based research, project management, decision-support 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. EKAT makes use of environmental information and environmental software that is on the Internet.

EKAT features include project management and organization capability, collaboration within project teams, and communication with other EKAT users through threaded discussions. EKAT may be used to assist in National Environmental Policy Act (NEPA) evaluations and to generate Programmatic Environment, Safety, and Occupational Health Evaluation (PESHE) reports, both of which are required by Department of Defense Instruction (DoDI) 5000.2. EKAT allows the user to archive supporting documentation by using EKAT tools to generate information or by uploading documents from outside EKAT into EKAT project files.

One of the most beneficial aspects of EKAT is its ability to serve as a preliminary environmental screening tool for regulatory requirements and other potential issues of concern. When you use EKAT, you may:

• perform screenings of chemicals used in a product or system, highlighting environmental and safety compliance issues for materials in your system;
• use the National Environmental Protection Act (NEPA) decision tree for your proposed operation to determine if an environmental assessment or environmental impact statement may be necessary;
• use the Emissions Calculator (EmisCalc) to estimate air emissions which may impact the need for a state air permit; and
• view information resource or report guidance documents, which provide additional topic information and links to resources for assistance with compliance requirements, environmental or safety and health issues, or other specific questions.

EKAT is currently being used at Kansas State University and by other individuals to assist with identification and evaluation of environmental issues.

Objectives and Vision

The vision behind the Environmental Knowledge and Assessment Tool (EKAT) is to serve society by developing a Web-based software tool that provides expert environmental management and regulatory compliance guidance to individual users, academia, government and the commercial business sector. EKAT was designed for use by people with little or no environmental background, as well as for use by environmental professionals. It is a Web-based product that can either run on an intranet, allowing no outside user access besides local personnel, or on the Internet.

EKAT is organized to act as both an active project management and review system, where environmental issues associated with a material or activity can be evaluated in a semi-automated process, and as a resource center where one would be able to conduct additional research on his or her own and quickly locate specific tools and other references to assist in evaluation efforts without having to filter through the magnitude of information that is available through a search of the World Wide Web.

All the information included inside EKAT's databases or linked to various research pages within the program was selected because it is considered to be from a reliable source. Databases, documents and other information found in EKAT are extensively reviewed and considered up-to-date for each new version of EKAT.

Applications and Audiences

EKAT development is funded by the federal government and managed by Marine Corps Systems Command (MARCORSYSCOM), and hence the primary focus of development is to assist MARCORSYSCOM with managing environmental issues for their projects and programs. While the current primary user group is the U.S. Marine Corps, EKAT is being developed with sufficient flexibility to be used in other applications. Within the Department of Defense, it can be used as an environmental resource for those involved in field activities, for those leading remediation efforts, and by environmental managers and engineers at military bases.
The EKA T development team, from the beginning, envisioned a broadly useful tool for not only the U.S. Marine Corps and the Department of Defense (DoD), but other federal agencies as well, including the Department of Energy (DOE), Environmental Protection Agency (EPA), Food and Drug Administration (FDA), Housing and Urban Development (HUD), Small Business Administration (SBA), U.S. Department of Agriculture (USDA), DoD General Counsel, and Environment, Health and Safety sections of U.S. Government agencies.

Various assessment and research features in EKAT are also useful to local and state governments, industry and business personnel, researchers, educators, and environmental consultants, as it is designed to provide information for users with differing levels of environmental knowledge. Business and industry environmental and leadership teams can use EKAT as a resource as they provide environmental stewardship and develop required reports and implement environmental management plans. Members of the regulatory community can use EKAT to find needed environmental information. Those who are working in health and safety, security, and emergency services often have a need for environmental information, which can be provided using EKAT. The public can make use of EKAT to find environmental information as well. In our work with communities, we have found that many citizens want to learn more about environmental chemistry and the risk associated with the contamination of their community.

Methods

A great deal of environmental, and health and safety information is available, and since the advent of the World Wide Web, accessing such information has become order of magnitudes easier and faster. One of the goals when we began developing the Environmental Knowledge and Assessment Tool (EKAT), since it is a Web-based software tool, was to make use of the plethora of useful tools and Web sites that are maintained by federal agencies, research groups, or other associations, without 'reinventing the wheel.' Some information needed to be brought within EKAT to enable some of the program features, which will be discussed shortly, while other information could be linked to from EKAT, allowing the user to have the most up-to-date information, since much of the content on these Web sites is updated on a regular basis.

What follows is a discussion outlining the main features of EKAT. Figure 1 shows the EKAT home page.
Figure 1: The Environmental Knowledge and Assessment Home Page

The EKAT software program is divided into six key areas, all of which can be accessed from the program’s home page (Figure 1):

- **Tools** – EKAT provides a number of built-in tools and immediate links to other to assist the user in evaluating environmental and health and safety issues, and find pollution prevention alternatives, including a units conversions tool to allow for easy conversion between different units of measure, a ‘materials screening’ feature to allow a user to quickly screen a single chemical for federal regulatory information and other safety or health issues without setting up a project, a ‘solvents screening’ feature for screening a chemical against regulated toxic air pollutant and hazardous waste lists (including those for seven selected states and access to other solvent regulatory information for these states), and easy access to particularly useful environmental tools created and maintained by others outside of EKAT.

- **Research** – The ‘Research’ section provides quick access to environmental resources allowing the user to research material properties, investigate environmental issues, and find pollution prevention alternatives. This section of EKAT includes links to Information Resource (IR) documents and research guides focusing on physical and chemical properties of materials, hazardous waste identification and management, and toxicology research.
Information Resources enable the user to find information about particular topics by giving an overview of the topic and providing links to Web sites with additional information for 70 different topics organized under the categories of environmental compliance, the National Environmental Policy Act (NEPA), safety and health, hazardous materials, explosives, and pollution prevention.

Other useful research links include pages explaining how chemical names for the same substance (synonyms) and Chemical Abstract Services (CAS) registry numbers may be found, how material safety data sheet (MSDS) information is organized; a link to the U.S. National Library of Medicine TOXNET Web site, a cluster of databases on toxicology, hazardous chemicals, and related areas; and a link to a periodic table and associated information provided by the Los Alamos National Laboratory.

• **Public Forum** – This feature allows EKAT users to share information they find useful and collaborate with other project team members in EKAT through the Public Project List, the Discussion Board, or by posting useful information in Public Documents. The information shared here can be seen by any peers or colleagues using EKAT.

The 'Public Project List' is a list of all projects that are chosen to be published by the project owner. Only the title of the project, date the project was created, and an e-mail contact of the owner are displayed. Users wishing to have more information about a particular project must contact the project owner.

• **Help** – The Help section provides assistance to EKAT users in several ways. Users can view the 'First Time User's Guide' or tutorials explaining various functions within EKAT. The 'First Time User's Guide', also available from the EKAT home page, gives an overall introduction to EKAT, as well as an overview of the various features available.

Also present under the Help section is contact information, including links to all appropriate state environmental agencies, as well as several United States Environmental Protection Agency (EPA) assistance hot-lines and Web sites for specific environmental or compliance issues, to help EKAT users answer specific questions they may have.

Finally, the 'Report Guidance' section found under 'Help' allows the user to view templates, instruction manuals, and guides for different types of environmental reports commonly used to document decisions about environmental issues or risk.

• **Search** – The search function allows the user to perform a quick text search for relevant documents and topics in EKAT, or to perform an advanced search of either EKAT or the World Wide Web. Search retrieves information from reports in each user's 'My Projects' files, as well as content from other pages in EKAT.
However, content from other user’s project files, including any documents uploaded into a project file, is not retrieved by Search.

- **My Projects** – The ‘My Projects’ and EKAT Project Manager features contained within this section allows multiple EKAT users to centrally organize and reliably document environmental solutions while working collaboratively to generate reports and make decisions. Under ‘My Projects’ one may opt to perform any of the following environmental assessments or features.

- **Environmental Screening.** The Environmental Screening assessment evaluates chemicals for federal environmental compliance issues and highlights key health and safety concerns for those chemicals. An EKAT screen preview of environmental screening results is displayed in a summary tabulated report (Figure 2). The program user can choose to transfer the tabulated results to a more-detailed report, which can be saved to a Word or PDF document.

- **Solvent Screening.** The Solvent Screening assessment evaluates solvent chemicals for state air and hazardous waste compliance while highlighting specific clean air act control technology requirements for solvent cleaning operations enforced by states. This assessment is currently available for the states of California, Georgia, Hawaii, Louisiana, North Carolina, South Carolina, and Virginia only.

- **TECCA (Tool for Estimating Chemical Concentrations in Air).** This tool may be used to estimate the average concentration in well-mixed air in a room where a volatile liquid chemical is released or spilled.

- **EmisCalc (Emissions Calculator).** EmisCalc estimates pollutant emissions associated with process activities, using EPA-approved air pollution factors (AP-42). Results are useful for air permitting requirements, and can also be seamlessly integrated with the modified TRACI assessment for lifecycle evaluations.

- **TRACI for EKAT.** The Tool for Reduction and Assessment of Chemical and other environmental Impacts (TRACI), developed by the EPA, has been adapted to EKAT. TRACI for EKAT allows the user to evaluate the environmental impact of chemical emissions to air or water over the multiple stages of the life of a product, and to make pollution prevention decisions based on this information.

- **NEPA (National Environmental Policy Act) Decision Tree.** The NEPA Decision Tree assessment outlines federally mandated requirements that federal agencies, including the U.S. Marine Corps, must fulfill before proceeding with a proposed action.
• **PESHE Report Generator.** The PESHE Report Generator creates a Programmatic Environment, Safety, and Occupational Health (PESHE) report using both, information input by the user and results of EKAT assessment reports for the project. This report can then be saved to a Word document for additional work.

**Figure 2: The Environmental Screening Results Summary Page**

**Conclusions**

The Environmental Knowledge and Assessment Tool (EKAT) is a Web-based research, project management, decision-support tool to help people identify, research, and evaluate environmental and safety-related issues. The program presents basic information on technical and regulatory requirements and is a preliminary screening tool for potential issues of concern. EKAT also serves as a resource center, with links to other references, tools, and databases to assist users in their research efforts.

Project manager features in EKAT allows multiple users to work collaboratively on environmental evaluations, and is particularly useful for people working together on one of the many assessment features available, such as the solvent or environmental screenings, the air emissions estimator, or the NEPA Decision Tree.
The resource features of EKAT provide a number of tools to assist in environmental evaluations, including information documents and guides, and links to environmental tools maintained by others outside of EKAT, such as the Environmental Protection Agency, the U.S. National Library of Medicine, the National Institute for Occupational Safety and Health, the Occupational Safety and Health Administration, the United States Fish and Wildlife Service, and many others.

Acknowledgments

Current Environmental Knowledge and Assessment Tool (EKAT) development is funded by congressional appropriation and managed by Marine Corps Systems Command (MARCORSYSCOM). The primary focus of development is to assist MARCORSYSCOM with complying with environmental regulations and evaluating environmental issues associated with weapons systems acquisition.

EKAT was designed and built by the team of M2 Technologies, Kansas State University, and CABEM Technologies as members of the National Environmental Evaluation and Remediation Consortium (NEER). M2 Technologies manages EKAT and provides application development and user support; Kansas State University provides subject matter expertise, application development, and content to the application; and CABEM Technologies provides the software development and application development expertise to the team.

The NEER consortium addresses complex environmental issues affecting the health and safety of our air, water, and land. Its focus encompasses environmental problems across the spectrum of federal, state, and local responsibilities. NEER accomplishes its mission through scientific research, environmental training, independent assessment, technology transfer, and community services/assistance with a unique teaming approach of integrating scientific and operational problem solvers. For questions about EKAT, please visit www.ekat-tool.com, e-mail Terrie Boguski or Ryan Green at ekat@ksu.edu, or call NEER at 800-798-7796.

References

Department of Defense Instructions (DoDI) 5000.2

Defense Environmental Network and Information Exchange
https://www.denix.osd.mil/denix/denix.html

Environmental Knowledge and Assessment Tool
http://www.ekat-tool.com

Environmental Protection Agency (EPA) – air emission factors and AP-42
http://www.epa.gov/ttn/chief/index.html
http://www.epa.gov/ttn/chief/ap42/index.html
Environmental Protection Agency – Tool for the Reduction and Assessment of Chemical and other environmental Impacts (TRACI)
http://www.epa.gov/ttn/chief/ap42/index.html

FAOSTAT
http://faostat.fao.org/

National Environmental Policy Act (NEPA)
http://ceq.eh.doe.gov/nepa/regs/nepa/nepaeqia.htm
http://www.epa.gov/compliance/nepa/
https://www.denix.osd.mil/denix/Public/Policy/Marine/5090.2A/contents.html

Pollution Prevention
http://www.epa.gov/p2/

Programmatic Environmental Safety and Occupational Health Evaluation (PESHE)

Solvent Alternatives
http://es.epa.gov/ssds/sagedown.html

Solvent Cleaning
http://www.epa.gov/ttn/chief/ieiip/techreport/volume03/iii06fin.pdf

State Environmental Agencies and Small Business Assistance Programs
http://www.smallbiz-enviroweb.org/sba/seasbapweb.html
In-situ Biostimulation for Remediation of Chlorinated Solvents in Groundwater: A Laboratory Study

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Abstract

Tetrachloroethylene or perchloroethene (PCE) is employed extensively in dry cleaning and carbon tetrachloride (CTC) has been used as fumigant in grain storage facilities. Remediation feasibility studies are being conducted using mesocosm experiments; a chamber is divided into six channels, filled with soil, with plants grown on top. Each channel is fed with contaminated water near the bottom and the effluent is collected, simulating groundwater flow conditions. The contaminants were introduced starting March 12, 2004. PCE is introduced at a concentration of about 2 mg/L (≈12 μmol/L) in three channels, two of them with alfalfa plants and the other with grass. CTC is introduced at a concentration of about 2 mg/L (≈13 μmol/L) in the other three channels, two of them with alfalfa plants and the other with grass. Since no degradation products were found at the outlet after about 100 days, one channel each for PCE and CTC with alfalfa was fed 1 L of 0.2% glucose solution. The glucose solution was fed once every month starting July 1, 2004 until February 2005. From October 1, 2004, one liter of 0.1% emulsified soy oil methyl esters (SOME) was fed to two other channels (with alfalfa), one exposed to PCE and another exposed to CTC, on the same addition dates as for glucose. The outlet liquid of the channel fed with PCE and SOME started to show some of the degradation compounds of PCE; however, the extent of degradation was not as great as that of the glucose fed channel. A similar trend was observed in the CTC fed channels. Based on the results from this and other studies, remediation using biostimulation and bioaugmentation will be implemented for a PCE contaminated site in Manhattan, Kansas; this will be a demonstration project which will be carried to other similar contaminated sites throughout Kansas.

Introduction

Chlorinated solvents have been widely used as degreasers in various industries and as fumigants in grain storage facilities. Past disposal methods and handling practices for chlorinated solvents have contributed to widespread contamination in soil and groundwater. In the United States, soil and groundwater at approximately 400,000 sites are contaminated with chlorinated solvents (Sutphin, 1996). Tetrachloroethylene (PCE) and trichloroethylene (TCE) were the fourth and second most frequently detected organic pollutants at U.S. National Priorities List (NPL) or Superfund sites, with PCE identified at 771 and TCE identified at 852 of the 1430 NPL sites as of September 1997 (US EPA, 1998).

In-situ bioremediation is used as an alternative to such traditional methods as groundwater pump-and-treat for treating groundwater contaminant plumes. This involves stimulating indigenous bacteria by adding electron donors and/or nutrients to the subsurface to increase bacterial growth yielding faster degradation rates. A variety of electron donors such as acetate, lactate, methanol, molasses and vegetable oils have been used for biostimulation of microbes that degrade chlorinated solvents (Grindstaff, 1998, Harkness et al, 1999).
Contaminated Sites

PCE has been deposited by dry cleaning operations in Manhattan, KS. Public supply wells are located about one mile down-gradient of the contaminated zone. According to the source investigation report (Terracon, 2004), PCE is primarily limited to the source areas; however, trichloroethylene (TCE), a degradation product of PCE, was also detected in the groundwater, at concentrations exceeding its MCL (5 µg/L), up to 0.75 mile east of the source. Concentrations of PCE from monitoring well samples were found to be as high as 26 mg/L at the source and 7.5 mg/L down-gradient (Terracon, 2004).

CTC contamination was detected by the EPA at the former Commodity Credit Corporation, United States Department of Agriculture (CCC/USDA) grain storage facilities at Murdock, NE. CTC is present in groundwater at concentrations exceeding 400 µg/L (Argonne National Laboratories, 2004).

Table 1. Physical/Chemical Properties of PCE and CTC.

<table>
<thead>
<tr>
<th>Property</th>
<th>PCE*</th>
<th>CTC#</th>
</tr>
</thead>
<tbody>
<tr>
<td>Molecular weight</td>
<td>165.83</td>
<td>153.8</td>
</tr>
<tr>
<td>Vapor pressure (mm Hg) at 25°C</td>
<td>18.47</td>
<td>115</td>
</tr>
<tr>
<td>Density (gm/cc)</td>
<td>1.62</td>
<td>1.594</td>
</tr>
<tr>
<td>Dimensionless Henry’s constant at 25°C</td>
<td>0.72</td>
<td>1.24</td>
</tr>
<tr>
<td>Solubility in water (mg/L) at 25°C</td>
<td>150</td>
<td>800</td>
</tr>
<tr>
<td>( \log K_{ow} ) and ( \log K_{oc} )</td>
<td>3.4 and [2.2-2.7]</td>
<td>2.83 and 2.04</td>
</tr>
<tr>
<td>Maximum Contaminant Level (MCL) in water (µg/L)</td>
<td>5</td>
<td>5</td>
</tr>
</tbody>
</table>


PCE (C\(_2\)Cl\(_4\)) Degradation Products: Trichloroethylene (TCE), Dichloroethylene (DCE), Vinyl Chloride (VC) & Ethene/Ethane/Methane.

CTC (CC\(_1\)\(_4\)) Degradation Products: Chloroform (CF), Methylene Chloride (MC), Chloromethane (CM) & Methane

Experimental System

A chamber is divided into six channels; each channel is 110 cm long, 65 cm high and 10 cm wide. The channels are filled with soil (up to 60 cm), and alfalfa is grown in channels 1, 2, 5 and 6, while fescue grass is grown in channels 3 & 4 (Figure 1). The inlet water is fed at 5 cm above the bottom of the channels. The contaminants were introduced starting March 12, 2004. PCE is 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 grass. CTC is introduced at a concentration of about 2 mg/L (~13 µmoles/L) in the other three channels, two of them with alfalfa plants and the other with grass. The depth of saturated zone in the channel is controlled by position of outlet tube (25 cm in this system). Plants are cut, at about 5 cm from the surface, and harvested at the beginning of each month.
Biostimulation by Glucose & Soy Oil Methyl Esters

Since no degradation was observed after 100 days, supplements were added for growth of microbes, to create anaerobic conditions and also for supplying hydrogen. One channel, each for PCE and CTC, with alfalfa was made anaerobic by adding one liter of 0.2% glucose solution. The glucose solution was fed once every month starting July 1, 2004 until February 2005. From October 1, 2004, one liter of 0.1% emulsified soy oil methyl esters (SOME) was fed to two other channels (with alfalfa), one exposed to PCE and another exposed to CTC. The SOME solution feeding was continued once each month until May 2005.

Analytical Method

Chlorinated solvents and methane were measured by gas chromatography (HP 5890 Series II, Wilmington, DE) equipped with a Flame Ionization Detection (FID) and a HP-1 column (Dimethyl Polysiloxane matrix, 30 m x 0.53 mm, Agilent Technologies). The injector temperature was set at 200°C and detector temperature was set at 300°C. Sample volume of 100 μl is injected in the column at 100°C and run for 5 minutes.

Results and Discussion

1. PCE

Figures 2, 3 and 4 show the inlet PCE, outlet PCE and degradation product concentrations for channels 1, 2 and 3. The outlet PCE concentration decreases after the addition of SOME (Figure 2) with conversion of PCE to TCE, DCE, VC and methane. Maximum concentrations reached are 2.8 μM TCE, 16.8 μM DCE, 1.1 μM VC and 4 μM methane. A carbon balance performed for inlet and outlet indicates more than 80% recovery.

Glucose addition leads to conversion of PCE mainly to DCE and not other degradation products (Figure 3). Breakdown of DCE is usually the rate limiting step in the degradation of PCE. The mean residence time in this channel (#2) varies between 2 to 7 days, which may not be sufficient time for the DCE to degrade further. However, almost all PCE is converted to DCE, unlike channel 1 (Figure 2), where more than 40% of the PCE remains in the outlet. Different microbial populations are being supported by the supplements, glucose and SOME. Methane is also observed in this channel in large amounts, most likely from glucose rather than from PCE. Even after stopping the feeding of glucose (day 328), PCE degradation continued to be carried out. Glucose could be stored as polysaccharides and cell materials and released slowly to supply the electron donor for dechlorination. After day 400, concentrations of outlet PCE and TCE increased and DCE decreased.

Since all of the inlet PCE was not transformed in channel 1, it is also 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 had a significant effect on the PCE and other product concentrations at the outlet. The concentration of PCE decreased from about 5 μM to 0.5 μM, TCE decreased from about 2.5 μM to 0.3 μM, DCE increased from about 5 μM to 17 μM and methane increased from about 0.5 μM to 4 μM (Figure 2).

In channel 3 (Figure 4), where no hydrogen donor was added, no degradation compounds were detected in the outlet and almost all inlet PCE came out in the effluent.
2. CTC

Figures 5, 6 and 7 show the inlet CTC, outlet CTC and concentrations of degradation compounds for channels 4, 5 and 6. Where no hydrogen donor was added (Figure 5), no degradation compounds were detected in the outlet and almost all inlet CTC came out in the effluent, as observed in channel 3 for PCE.

Figure 6 shows the CTC degradation in the glucose treated channel. Forty days after the addition of glucose, the outlet CTC started to decrease and reached a low conc ($< 2 \mu M$). Chloroform appeared, but never exceeded a concentration of $2.5 \mu M$. Methylene Chloride (MC) was also detected but stayed less than $1 \mu M$.

Figure 7 shows the CTC degradation pattern in SOME fed channel. Outlet CTC decreased within 40 days after first dose of SOME addition. Chloroform was not detected above a concentration of $2 \mu M$. However, methylene chloride (MC) increased and decreased regularly. This is due to the variation of the residence time of the inlet CTC. During the start of a month, the plants are harvested, and therefore, the evapotranspiration rate is less. In these days, most of the water flows out and therefore, the residence time is less. However, at the end of the month, when the plants are larger, the evapotranspiration rate is higher and the effluent is less. This leads to higher mean residence times and consequently, higher degradation of MC.

Conclusions

Supplements such as glucose and SOME stimulate the indigenous microbes and help degrade PCE & CTC. The conversion rate of inlet PCE was higher in a glucose fed channel compared to a SOME fed channel. Degradation compound ratios were not the same in the glucose and SOME amended channels. SOME fed at the inlet appears to be trapped in the initial portion of the channel. The outlet MC in SOME and CTC fed channel depends on the residence time of the inlet CTC.

Future work

KB-1, a consortium of Dehalococcoides and other organisms, will be introduced in the channel for complete degradation of PCE. Results from these and other studies will be used to implement a pilot study and later a full scale remediation in the field.

Acknowledgement

The Kansas Department of Health and Environment and the Kansas Agricultural Experiment Station provided financial support.
References


Figure 1. Side and top view of the channels: Channels 1, 2, 5 & 6 are alfalfa and channels 3 & 4 are grass. Lighting is provided by a pair of tube lights for each channel.
Figure 2. Inlet PCE and outlet PCE, TCE, DCE, VC & methane concentrations for channel 1. Water samples taken on indicated days after beginning (March 12, 2004) exposure. Soy Oil Esters added on days 203, 236, 266, 299, 328, 359, 387 & 415.

Figure 3. Inlet PCE and outlet PCE, TCE, DCE & methane concentrations for channel 2. Water samples taken on indicated days after beginning (March 12, 2004) exposure; Glucose solution added on day 110, 151, 173, 203 and 236, 266, 299 & 328.
Figure 4. PCE concentrations in inlet and outlet of channel 3. Water samples taken on indicated days after beginning exposure (March 12, 2004).

Figure 5. Inlet CTC and outlet CTC, CF, MC & methane concentrations for channel 4. Water samples taken on indicated days after beginning (March 12, 2004) exposure.
Figure 6. Inlet CTC and outlet CTC, CF, MC & methane concentrations for channel 5. Water samples taken on indicated days after beginning (March 12, 2004) exposure. Glucose solution added on day 110, 151, 173, 203 and 236, 266, 299 & 328.

Figure 7. Inlet CTC and outlet CTC, CF, MC & methane concentrations for channel 6. Water samples taken on indicated days after beginning (March 12, 2004) exposure. Soy Oil Esters added on days 203, 236, 266, 299, 328, 359, 387 & 415.
Microcosm Investigation of the Dechlorination of Tetrachloroethene

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Abstract

Tetrachloroethylene (also known as perchloroethene or PCE) is widely used as a dry cleaning solvent and a degreasing material. It is among the most abundant groundwater contaminants at many industrial, residential and military sites. PCE may be present as a dense non-aqueous phase liquid that tends to settle in ground water creating a contaminated plume, which may endanger public water wells. Aerobic degradation of PCE is ineffective; however, under anaerobic conditions dechlorinating bacteria use PCE as a terminal electron acceptor, and sequentially dechlorinate PCE to less chlorinated ethenes.

Our study site is located in Manhattan, Kansas were PCE was used in a former dry cleaning facility. The contaminated plume is moving toward the city public water wells. Therefore, the Kansas Department of Health and Environment is collaborating with Kansas State University in characterizing and remediating this site. Previous work done by a contractor shows that PCE and its degradation products such as trichloroethene (TCE), cis-1,2dichloroethene (c-1,2-DCE) and vinyl chloride (VC) are also present in the ground water plume above their maximum contamination levels. The objective of our study is to use microcosms for evaluating biostimulation (addition of nutrients) and bioaugmentation (addition of microbes) of endogenous soil and water microflora that can carry out reductive dechlorination of PCE.

Microcosms from the site groundwater were amended with nutrients. Different concentrations of yeast extract with either lactate or soy oil methyl esters were tested to enhance reductive dechloronation beyond c-1,2-DCE. Chlorinated ethenes were measured in the gas phase of these microcosms using gas chromatography. This project is still under investigation and preliminary results are presented. Data shows that higher concentration of nutrients supported faster degradation of PCE. It took 7 days when the microbial culture KB-1 was added, compared with weeks in the microcosms without KB-1. Small amounts of ethene and much methane were also detected.

Keywords: Biostimulation, Bioaugmentation, PCE, Microcosms

Introduction

The soil and ground water of the Cinderella Dry Cleaner site in Manhattan, Kansas was contaminated after about 30 years of operation. Perchloroethene (PCE) which was used as the primary cleaning solvent was found in the soil system. In the soil
PCE is subject to evaporation into the atmosphere, leaching to groundwater and sorption to soil (Sirem Laboratories, 2004). The main effects of PCE in humans are neurological, kidney and liver dysfunction, irritation in upper respiratory track as well as the eyes. It may also increase incidences of several types of cancer (EPA-Air Toxics, 2004). Studies showed that natural and enhanced bioremediation technologies have proven to be powerful approaches for remediating chlorinated solvents, including PCE. Several anaerobic microbes have been shown to metabolize PCE in a process called dehalorespiration. Thus far, Dehalococcioids spp. are the most important microbial populations involved in the appearance of benign end products, methane and ethene (Lendvay, et.al. 2004). In the city of Manhattan, public water wells are located 1.5 miles east of the contaminated zone and are at risk of contamination because of a ground water gradient from west to east. Several monitoring wells were installed along the plume to take water samples. PCE is present at the source area at a concentration of 26 ppm. Biodegradation products were detected above maximum contamination levels downgradient (Bruggeman, 2004). Using CPT probes and well cores, the soil to 45 ft below ground surface was clayey above the sand in the aquifer. The purpose of this study is to investigate whether biostimulation and bioaugmentation will stimulate the degradation of PCE. Microcosms were prepared with contaminated water taken from the site and treated with different amendments. The efficiency of the bacterial culture KB1 was also tested in these microcosms. Biodegradation products (Fig.1) were detected in the gas phase of these microcosms.

![Chemical structures of PCE, TCE, VC, and Ethene](image)

**Fig.1.** Sequential reduction of PCE to ethene by anaerobic reductive dechlorination.

**Experimental System**

**Microcosms:** aqueous microcosms were prepared in 16 ml sterile glass bottles. Contaminated water from monitoring well number 5, the closest well to the source was collected and used in this set of microcosms since it has the highest concentration of PCE. Final volume of site water and treatment ingredients was 12 ml. Rezasurine was used as an indicator for oxygen availability. When the color changes from blue to pink to colorless this indicates a change from aerobic to anaerobic conditions. The head space in microcosms were flushed with argon gas to displace the air and quickly sealed with mininert Teflon screw caps. (Fig.2) KB1 was added to some treatments after anaerobic conditions were established in the microcosms. Microcosms were sampled at several time intervals. Hundred µl samples of headspace gas were injected into a gas chromatograph.
Analytical Methods
Gas chromatograph (HP5890 Series II) with flame ionization detection (FID) using an HP1 (dimethyl polysiloxane matrix) column was used for headspace analysis of samples; sample volume was 100μl.

Results and discussion
Results in Figures 3-6 show that both lactate and soy oil methyl esters stimulate reductive dechlorination of PCE. In the presence of bacterial culture KB1, faster degradation rates are observed as shown in Fig.3a and Fig.4a. In this study we tried different concentrations of nutrients and noticed that lowering the concentration of lactate and soy oil methyl esters would decrease the biostimulation effect and delay PCE removal in presence of KB1. The same effect is seen when yeast extract is lowered to 0.001% or 0.003%, See Fig.5 and Fig.6. Large amounts of methane are produced while cDCE appears at the beginning but later on the levels of cDCE dropped down in both lactate and soy oil methyl ester treatments with KB1; See Fig. 5b and Fig 6b.

Conclusions
Biostimulation helps in degrading PCE. Bioaugmentation with KB1 completely removed PCE within about a week. Nutrient availability ensures faster and more complete degradation. It seems that yeast extract and soy oil methyl esters are a promising combination for enhancing microbial degradation.

Future Work
The knowledge gained from these results will be used in a pilot study at the site and some nutrient combination of yeast extract, soy oil methyl esters and glucose will be injected.
The bacterial culture (KB1) will also be introduced to the site followed up by routine sampling from wells down gradient to test for degradation products.

Acknowledgment
This study is financially supported by Kansas Department of Health and Environment. The microbial consortium (KB1) was kindly provided by SiRem Company. The authors would like to thank R. Karthikeyan for helping in GC analysis and Danielle Ngaba and Tanner Callender for assistance in GC operation.

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Fig. 3. PCE degradation when lactate was used as a substrate A. with KB1 and B. without KB1.
Fig. 4. Degradation when Soy oil methyl ester (Soy oil ME) was used as a substrate A. with KB1 and B. without KB1.
Fig. 5. Effect of Lactate and Yeast Extract Concentration. A. without KB1, B. C., and D. with KB1.
Fig. 6. Effect of Soy oil Methyl Ester (Soy oil ME) and Yeast Extract Concentration. A. without KB1, B., C. and D. with KB1.
Effect of blending with Polycaprolactone on the anti-bacterial properties of Chitosan for Periodontal Tissue Engineering

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ABSTRACT

The aim of this work was to evaluate the anti-bacterial properties of chitosan and chitosan/polycaprolactone blend membranes against oral pathogens *Streptococcus mutans* and *Actinobacillus actinomycetemcomitans*. Membranes were suspended in bacterial cultures and incubated for 24 hours. The bactericidal effect (by optical density measurements and agar plating) and bacterial adhesion (by digital imaging and scanning electron microscopy) of the membranes were studied.

Results indicated that the presence of chitosan decreased the growth of both organisms in culture when compared to control (no membranes). This property was gradually compromised by the addition of PCL. SEM images indicated minimal presence of bacteria on chitosan. The adherence of bacteria increased steeply in 25%, 50% and 75% PCL respectively and pure PCL was almost covered with a biofilm-like layer of bacteria. Agar cultures indicated that chitosan does not allow bacterial viability and proliferation on its surface. In conclusion, the anti-bacterial properties of chitosan are compromised by blending with PCL, despite other advantages for tissue engineering applications.

1. INTRODUCTION

The feasibility of tissue engineering¹ and other techniques such as guided tissue regeneration, bone grafting and the use of enamel matrix derivatives have been demonstrated by restoring all three tooth-supporting tissues i.e. periodontal ligament fibers, alveolar bone and the cementum²,³. Infection by microorganisms is a common post-operative detrimental factor to the success of these implants⁴. Therefore, the unique challenge in oral tissue regeneration is abating the colonization of pathogens while promoting the colonization of fibroblasts. The search for ideal biomaterials is still on going where the properties of the matrices are dictated concurrently by a number of factors such as degradation kinetics, mechanical stresses at the site of implant, the cell-material interactions, and anti-bacterial activity⁵.

Chitosan is a positively charged polysaccharide with well-known anti-bacterial and biodegradable properties explored in wound dressings, food packaging and cartilage tissue regeneration⁶. It is hydrophilic and soluble in weak aqueous acids (pH<6.3); supports the
adhesion and viability of variety of cell types. The biological activity and lysozyme-mediated
biodegradation of chitosan can be improved by blending with gelatin and amorphous poly(lactide-co-glycolide) respectively.

The mechanical and biological properties of chitosan can also be improved by blending with polycaprolactone (PCL), an FDA approved semi-crystalline polyester. PCL has been widely investigated in sutures, catheters and drug delivery due to its hydrophobic nature, neutral charge and easy processing conditions (T_g -60°C and T_m 60°C).

Focus of this work was to evaluate the effect of blending on bacterial cell-material interactions. Specifically, the bactericidal and prohibitive capacity of chitosan/PCL blend membranes against bacterial proliferation and adhesion were studied. This study was conducted using oral pathogens Streptococcus mutans (Gram-positive, facultative anaerobe, spherical, leading cause of dental caries) and Actinobacillus actinomycetemcomitans (Gram-negative, facultative anaerobe, rod shaped, causes juvenile periodontitis).

2. MATERIALS AND METHODS

S.mutans (ATCC 25175, NCTC 10449) and A.actinomycetemcomitans (ATCC 43719, NRRL 2501) were grown aerobically in brain heart infusion (BHI) broth at 37°C for 48h. Cultures in exponential growth phase were used for all experiments. Chitosan (~85% degree of deacetylation, molecular weight MW>310kD) and PCL (MW 80kD) were purchased from Sigma Aldrich, St. Louis, MO.

2.1. Formation of blend membranes: Three milliliters of chitosan solutions (2% w/v) in 0.5M acetic acid were added drop-wise to 10mL of PCL solutions of different concentrations in glacial acetic acid to obtain blends of 25%, 50% and 75% PCL composition by mass, respectively. The mixtures were slightly warmed and stirred to form homogenous solutions, poured into Teflon Petri dishes and dried for 18h in an oven at 55°C to form uniform membranes. Membranes were cut into 2cm x 2cm samples, neutralized and sterilized by immersion in 25mL of 90% ethanol each, for 15min and washed thoroughly in sterile phosphate buffered saline (PBS).

2.2. Analysis of bacterial proliferation in suspension: Samples were suspended in 5mL of bacterial broth of known optical density (OD) taken in glass vials with rubber caps and incubated at 37°C with constant, gentle shaking. Cultures without any membranes were taken as control. At various time points, 1mL of each culture was retrieved and optical density was measured using a spectrophotometer at 600nm. After 24h, the matrices were removed and analyzed for bacterial adhesion. For this purpose, samples were fixed using 3.7% paraformaldehyde followed by rinsing with sterile water and ethanol. Subsequently, samples were air-dried in a vacuum

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desiccator, sputter-coated with gold and imaged using a JEOL 6360 Scanning Electron Microscope (Jeol USA Inc., Peabody, MA).

2.3. Contact-dependent anti-bacterial property of chitosan surface: Dense ‘mats’ of bacteria were generated on BHI agar plates by spreading 25μL of turbid bacterial cultures over approximately 2cm × 2cm area and incubating at 37°C for 12h. Chitosan and PCL blend membranes from section 2.1 were placed on these mats and further incubated. Visible changes in the morphology of the mats underneath the membranes were monitored with respect to control (mats with no membranes on top) and digital images were obtained. After 24h, membranes were rehydrated by adding few drops of media, gently removed and placed on a clean agar plate, bottom side facing down. Bacterial growth accompanying the membranes was monitored as before.

2.4. Statistical analysis: All experiments were repeated three or more times with triplicate samples in each experiment. Significant differences between experimental groups were evaluated using a one-way Analysis of Variance (ANOVA) with 99% confidence interval.

3. RESULTS AND DISCUSSION
Transient changes in optical density (Figure 1) of bacterial broths with suspensions of chitosan and blend membranes indicated that chitosan showed the least decreased growth of bacteria in suspension when compared to control. There were no significant differences in the optical densities of 25% PCL and 75% PCL suspensions. Nevertheless, the growth of bacteria in presence of these membranes indicated that chitosan, PCL or their blends were not bactericidal to either of these Gram-positive and Gram-negative bacteria. SEM images indicated negligible adhesion to both strains of bacteria to chitosan membranes (Figure 2). The adhesion increased

![Figure 1: Presence of chitosan decreased growth of S. mutans and A. actinomycetemcomitans.](image-url)
drastically with addition of PCL which is known to be susceptible to bacterial action. Bacteria not only completely colonized the PCL membranes but strongly adhered to them.

The anti-microbial properties of chitosan have been attributed to its cationic nature. The net positive charge binds the negatively charged bacterial cell walls and breaks it, resulting in cell death\(^1\). Therefore, to study the contact dependent anti-bacterial action of chitosan and its blends, bacterial mats were allowed to grow in direct contact with membranes on agar plates (Figure 3). After 24 hours, there was no growth accompanying chitosan membranes which retained their transparency. On the blend and PCL membranes, growth spread to the surroundings of the membranes, although changes underneath the membranes could not be examined due to their opaque nature. The membranes were rehydrated and peeled off to expose voids suggesting that the bacteria adhered to the membranes. To test the viability and proliferative ability of the adherent bacteria, peeled membranes were transferred to a fresh agar plate and observed for growth. Growth was similar to original agar plate with mats on which membranes were seeded.

These results suggest that chitosan which allows minimal adhesion to Gram-positive or Gram-negative bacteria on its surface does not allow the proliferation of the adherent bacteria implying that they were not viable. Further investigations into the viability of adherent bacteria using LIVE/DEAD fluorescent stain would provide useful insights into the contact-dependent anti-bacterial properties of chitosan surfaces.
4. CONCLUSIONS
It is not apparent that chitosan kills bacteria but proliferation is lower in comparison to blends. Bacterial adhesion and proliferation is also negligible on chitosan surface which increases with PCL content. Anti-bacterial properties of chitosan are compromised by blending with PCL.

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6. REFERENCES


Flow perfusion improves seeding efficiency and spatial distribution in scaffolds for bone tissue engineering

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ABSTRACT: Scaffold seeding with osteoblastic cells affects the development of bone grafts, determining initial cell number and cell spatial distribution in the scaffold. Spinner flasks and rotating wall vessels have been used as dynamic systems, but improvement is desirable. We evaluate the effect of perfusion on seeding efficiency and spatial distribution of osteoblastic cells in polymeric scaffolds with different architectures. The effect of fiber size and surface modification by oxygen plasma treatment is also evaluated. MC3T3-E1 cells were seeded statically and in a flow perfusion bioreactor. Dynamic seeding resulted in the most efficient seeding technique, and improved the distribution of the cells throughout the scaffold surface when compared to the static seeding.

1 INTRODUCTION

The main goal of bone tissue engineering is the creation of artificial constructs to repair or simply replace lost or damaged osseous tissue. Common tissue engineering strategies involve the extraction of cells from a small piece of tissue for in vitro expansion. This culture can be carried out in a three dimensional scaffold that allows the formation of new tissue after implantation (Bonassa et al. 1998). Most approaches in this field are based on common bioactive factors, consisting on cells (generally stem cells or osteoblastic cells), a scaffolding material, and growth and differentiation factors (Boden 1999). Furthermore, the in vitro creation of an efficient construct can be accelerated by applying certain mechanical and chemical stimuli that can elicit specific responses to the cells.

Chemical stimulation is carried out by using growth and differentiation factors specific for different responses. Growth factors play a major role in cell division, matrix synthesis and tissue differentiation (Lieberman et al. 2002). Examples of these proteins are bone morphogenetic proteins (BMPs), which have been demonstrated to induce the differentiation of mesenchymal stem cells into an osteoblastic lineage (BMP-2 and BMP-7), and the vascular endothelial growth (VEGF-β1) factor that greatly enhances angiogenesis (Wang et al. 1990, Trivedi et al. 1999). The need for in vitro mechanical stimulation in tissue engineering is drawn from the fact that most tissues function under specific biomechanical environments in vivo. These enivronments play a key role in tissue remodeling and regeneration. The stresses can be translated into different kinds of forces that range from load bearing to hydrodynamic forces due to fluid flow (Stolz et al. 2009). Thus, the mechanochemical microenvironment that progenitor cells grow into controls the fate of these cells while undergoing differentiation.

In many tissue engineering applications, bioreactors are used to impart certain forces that imitate different mechanical stimuli occurring in the body, thereby enhancing the formation of an extracellular matrix similar to the in vivo matrix. Moreover, bioreactors must meet other requirements in order to create grafts that, when implanted, will lead to the regeneration of damaged organs. They have to efficiently transport nutrients and oxygen to the construct, maintaining an appropriate concentration in solution. In general, a scaffold is seeded with cells and supports the formation of extracellular matrix (ECM). Thus, the bioreactor should induce a homogeneous cell distribution throughout these structures. Tissue engineering bioreactors can be used for cell seeding and/or long term cultures.

The first step to culturing cells in a three dimensional environment is the seeding of scaffolds (Martin et al. 2004). Along with the characteristics of the material, this process plays a crucial role in the formation of efficient constructs. Scaffold seeding determines the initial number of cells in the construct, as well as their spatial distribution throughout the matrix. Consequently, proliferation, migration and the specific phenotypic expression of the engineered tissue will be affected by the utilized seeding tech-
tions were tested for dynamic seeding in a flow perfusion system (Li et al. 2001). Static seeding techniques have resulted in low efficiencies and poor cell spatial distribution (Li et al. 2001, Burg et al. 2000, Holy et al. 2000). A low yield diminishes the development of specific functions related to cell-cell interactions and increases the amount of cells required; therefore, the usage of new seeding techniques becomes imperative.

In order to address these issues, researchers have incorporated convection into the process of cell seeding, suppressing some of the mass transfer limitations encountered in the static procedure. Spinner flask bioreactors have been implemented to create convection and, thereby, hydrodynamic forces that could help increase mass transport. Nevertheless, in these systems, most of the cells are still found towards the outer surface of the scaffolds (Vunjak-Novakovic et al. 1998). This behavior may be due to the poor convection to the interior of the scaffold, making migration the only way for cells to reach the interior.

One way to guarantee mass transfer to the interior of the scaffold and a better distribution of cells is by applying perfusion (Burg et al. 2000, Wendt et al. 2003, Martin et al. 2004). In this technique, the construct is press fitted into a chamber, and the cell suspension is flowed through it (Bancroft et al. 2003). Li et al., used a depth filtration system to seed poly(ethylene terephthalate) matrices at a rate of 1ml/min. The cell suspension was recycled to increase the yield. Cell density increased along with the inoculation cell number, the yield stayed constant and lower than that achieved with the filtration (Li et al. 2001).

Given the fact that most mass transport limitations are diminished in a flow perfusion system and the possibility to perform several cycles of infiltration of the scaffold with a cell suspension, it can be hypothesized that seeding scaffolds in a flow perfusion system will yield high efficiencies and homogeneous distributions of cells throughout the construct. In this study, we evaluate the effect of flow perfusion on seeding efficiency and cell spatial distribution on fibrous polystyrene matrices and foams seeded with osteoblastic cells, using a flow perfusion system also suitable for long-term cultures. Furthermore, the effects of fiber size and surface modification by oxygen plasma were also assessed.

2. MATERIALS AND METHODS

Scaffolds

Two scaffold architectures used for bone tissue engineering and other tissue engineering applications were tested for dynamic seeding in a flow perfusion bioreactor (figure 1). Fibrous polystyrene matrices with fiber diameters of 20, 35 and 50μm obtained by melt blowing were used. These fibers were also treated in an oxygen-plasma cleaner (Harrick), for 1 min at medium intensity and a pressure of 200mmHg, in order to increase their hydrophilicity and improve cell-matrix interactions.

Foams prepared by particulate leaching, using sodium chloride as the porogen, were also utilized for seeding. The grain size of the NaCl was 300μm. Briefly, PLLA (MW 10,000) (Birmingham Polymers) was dissolved in chloroform at a concentration of 5% w/v. The solution was then poured on a sodium chloride bed, and the solvent was allowed evaporate for 2 days. The salt-polymer composite was molded at 500 psig and 120°C and cut into discs. Salt leaching was performed using deionized water for 3 days. All scaffolds had a porosity of 95%, with a diameter of 8 mm and thickness of 3 mm.

Cell Source

Osteoblastic MC3T3-E1 cells were utilized. Cells were cultured on modified essential medium (D-MEM) in T-75 culture flasks. Media was changed every other day. Prior to seeding, cells were lifted using trypsin with a standard procedure and counted using a hemacytometer. Seeding inoculation numbers were 1.25x10^5, 2.50x10^5, 5x10^5 and 1x10^6 cells.

Seeding Techniques

a. Static Seeding: Scaffolds were pre-wet with 200-proof alcohol under vacuum, rinsed in phosphate buffered saline (PBS) and placed in a low-attachment well plate prior to the seeding. Cells suspended in dulbecco’s modified essential medium (D-MEM) were slowly distributed on top of the scaffolds and allowed to attach for 2h. The wells were then filled with medium and cells were allowed to condition for 8h, in an incubator at 37°C and under 5% CO2.

b. Shearing of scaffolds seeded statically: Scaffolds were seeded statically for 2h, as explained previously, and allowed to condition for 4h. Constructs were then subjected to flow in the perfusion bioreactor (figure 1) at a flow rate of 150 μl/min 8h. Briefly, the system was cured with DMEM, and the scaffolds were placed in the perfusion chamber. Flow was incorporated using a peristaltic pump.

c. Dynamic seeding: The flow system was cured with DMEM for 2 h. Pre-wet scaffolds were press-fit in the flow perfusion chambers of the bioreactor. Cell suspensions were poured on top of the scaffolds
using a micro-pipette, and flow was then incorpo­
rated at 150 µl/min. Cells were pumped up and
down through the scaffolds for 2h by changing the
direction of the pump every 5 min. Cell conditioning
was allowed statically for 2h, and flow was re­
incorporated at 150 µl/min for 8h.

Cell quantification

Cellularity of the scaffolds was determined using
a PicoGreen assay (Molecular Probes) after perform­
ing three freeze-thaw cycles to burst the cells.

Histological Analyses

Seeded scaffolds were infiltrated under vacuum
and fixed in buffered formalin (3.7% v/v) for 24h.
Fixed constructs were dehydrated in a graded series
of ethanol solutions with increasing concentrations
and a final with 100% clear-rite (Electron micros­
copy science). Each step was carried out under vac­
uum for a period of 10 min for proper infiltration of
the dehydrating solutions into the scaffolds. Dehy­
drated samples were then infiltrated with solutions
of increasing concentrations of SPUR resin in etha­
nol; each step was carried out under vacuum for a
period of 2h. Finally, the samples were embedded in
SPUR for 12h at 80°C. Samples were sectioned in 3-
µm sections in a microtome. Staining was performed
with toluidine blue. Microscopy images were cap­
tured with Metamorph©.

3. RESULTS AND DISCUSSION

In figure 1, different seeding techniques are com­
pared at different fiber sizes and an inoculation
number of 1.25x10^5 cells. In most cases, perfusion
seeding yielded the highest number of cells attached
to the scaffold. A significant number of cells were
detached from the statically seeded scaffolds when
these were sheared under flow in the perfusion
system. Therefore, even in cases in which static seeding
yielded the highest cellularity, the number of stati­
cally seeded cells that remained attached to the scaf­
fold after shear was lower than that achieved under
dynamic conditions.

![Figure 1. Effect of seeding technique and fiber size on the number of cells attached per scaffold using an inoculation number of 1.25x10^5 cells. All scaffolds were oxygen plasma treated.](image)

![Figure 2. Effect of cell inoculation number on the seeding efficiency of (a) untreated scaffolds and (b) oxygen-plasma-treated scaffolds at different seeding techniques. All scaffolds had a fiber size of 20 µm](image)

The efficiency of seeding, defined as the percent­
age ratio of the number of attached cells to the in­
oculation number, is shown in figure 2 for the dif­
ferent seeding methods. The efficiencies achieved
with the different techniques in untreated polysty­
rene fibers are compared in figure 2a. As a general
trend, it is observed that seeding efficiency de­
creases at higher inoculation cell numbers. As men­
tioned previously, dynamic seeding yielded the highest efficiency in most cases, with a maximum value of about (56±5) %. Oxygen-plasma treated fib­
rous scaffolds (Figure 2b) had a similar trend on
seeding efficiency with respect to inoculation number and seeding technique. Nonetheless, much higher efficiencies were obtained after the treatment, achieving efficiency values of up to (87±18) %.

In the dynamic seeding, the cellular inoculate is passed through the scaffold several times. Thus, during this process, cells have more opportunities of attaching and may be able to find an optimal place for attachment. This phenomenon could explain why the dynamic seeding resulted in the most efficient technique. Treatment with oxygen plasma incorporates hydroxyl groups on the surface of the PLLA, making it more cell friendly. Cell receptors will interact with the hydroxyl groups and induce a stronger cell attachment. Therefore, the number of cells attached to the oxygen-plasma-treated scaffolds is greater than the number of cells attached to the unmodified polymer.

The dependence of the scaffold cellularity with the inoculation number after dynamic seeding is presented in Figure 3. Cell attachment increased with the inoculation cell number, but it plateaus at an inoculation number of 5x10^5 cells. This indicates that the surface is saturated at a certain number of cells. After 5x10^5 inoculated cells, all spaces available for attachment are occupied, and thereby, after that point, the scaffold cellularity is constant and independent on the inoculation cell number.

There is not a significant difference between fiber sizes of 20 and 35µm; however, fibrous matrices with 50µm fibers had a significantly lower number of cells attached. Nonetheless, when scaffold cellularity is normalized with the surface area available for attachment (Figure 4), the trend observed is changed. This normalization is referred to as cell surface density, and it keeps a similar dependence on the inoculation cell number to that of the cellularity. However, the surface density observed in the 20-µm fibers was significantly lower than those obtained with larger fiber sizes. This effect is related to the dimension of the fibers and the cells. MC3T3-E1 cells have an approximate size of 20µm; thus, it is easier for them to interact with a surface that is reasonably larger than they are.

Figure 3. Dependence of the number of cells attached per scaffold on the cell inoculation number and fiber size of (a) untreated scaffolds, and (b) oxygen-plasma-treated scaffolds after flow perfusion seeding.

Figure 4. Dependence of cell surface density on cell inoculation number and fiber size of (a) untreated scaffolds, and (b) oxygen-plasma-treated scaffolds after flow perfusion seeding.

Figure 5 shows the dependence of cell surface density with scaffold architecture. In the foams, cell surface density increased with the inoculation number, a trend similar to that observed with the fibrous matrices. However, having dimensions and porosity similar to those of the fibrous matrices, the foams yielded lower cell densities. Consequently, scaffold architecture directly affects cell attachment in a flow perfusion system.
Hystological analysis (data not shown) of scaffolds seeded statically demonstrate that most of the cells were localized towards the periphery of the scaffolds, with very few cells present in the center. In the dynamically seeded scaffolds, on the other hand, comparable cell numbers were observed both at the periphery and the center of the scaffold, demonstrating that flow perfusion seeding improves cell homogeneity throughout the scaffold surface.

4. CONCLUSIONS

Incorporation of flow perfusion improves seeding efficiency and cell attachment. The treatment of polymeric scaffolds with oxygen plasma greatly improves cell-matrix interactions. However, trends on cell attachment with respect to fiber sizes and seeding technique persisted. Furthermore, cell spatial distribution is improved by the incorporation of flow perfusion in the seeding stage.

5. REFERENCES


Tocopherols and CEHCs Modulate Platelet Thrombus Formation

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1. Introduction

According to the American Heart Association, cardiovascular disease (CVD) is one of the leading causes of death in United States. Platelet activation and aggregation plays an important role in thrombus formation which leads to CVD. Many studies have reported that high intake of vitamin E (α-tocopherol), a major nutritional supplement and an antioxidant, reduced the risk of cardiovascular disease where as the high intake of other antioxidants (vitamin C and beta carotene, butylated hydroxytoluene) did not have any effect indicating that vitamin E possess non-antioxidant properties in reducing the risk of cardiovascular disease by inhibiting platelet adhesion and aggregation in vitro ¹,².

Angelo et al and Freedman et al³,⁴ also suggested that αT possess non-antioxidant properties. This has been supported by the studies done using the oxidized form of αT (which is devoid of antioxidant property), which also has considerable effect on inhibiting platelet aggregation². Williams et al showed that platelets produce functionally significant amounts of NO and that NO produced inhibits platelet adhesion. Freedman et al reports that αT increases nitric oxide (NO) production by adenosine di phosphate (ADP) activated platelets, by inhibiting protein kinase C activation and attenuating the super oxide release in a way independent of NO production ⁴,⁵.

Previous studies have shown that the effect of αT is pronounced only when it is incorporated into platelets ²,⁵. Supra physiological levels of αT (~1mmol/L) are required to achieve these effects ²,⁴. Recent clinical studies have not shown any favorable effects of αT on cardiovascular disorders. It has been shown that oral supplementation of vitamin E reduces platelet adherence to collagen coated slides ⁵ and these studies are done using platelet rich plasma (PRP) with ADP as aggregating agent. However, it has been shown that physiological levels of ADP do not aggregate platelets all by themselves in most of the cases¹¹. It has been shown that γT also possesses anti-aggregating properties besides antioxidant properties⁶. However there are no studies demonstrating the effect of γ-Tocopherol (γT) or other metabolites (α and γ carboxy ethyl hydroxyl chromans (CEHCs)) of tocopherols on platelet adhesion. Structure of tocopherols and their metabolites are presented in Figure 1.
In order to understand the direct affect of tocopherols and their metabolites on platelet aggregation and platelet–collagen interactions, a parallel plate flow chamber model is used, where whole blood is perfused on the collagen coated surface for 5 minutes and the % platelet coverage on the surface of collagen is quantified. The effect on platelet–platelet interactions are studied in a platelet aggregometer, where the aggregation is induced by convulxin. The mechanism by which these tocopherols act on platelets is still not known. The main aim of our study is to understand the effect of tocopherols and CEHCs on the initial stages of platelet aggregation and propose the underlying signaling mechanism through which these drugs may affect the platelets.

2. Materials and Methods

Flow Experiments

Blood samples are collected from healthy donors (30-60 mL, depending on shear rate) into BD syringes containing low molecular weight heparin (LMWH) as anticoagulant (40 U/mL). Mepacrine (10 μM), fluorescent label is then added to blood and is allowed to incubate for 10-15 minutes. All donors gave informed consent to participate in our study according to methods approved by the University of Oklahoma Institutional Review Board.

The arterial flow environment is modeled with an in vitro parallel plate flow chamber. The dimensions of the flow channel are 0.0127 x 0.6 cm.

αT (20 and 50 μM), γT (20 and 50 μM), αCEHC (1, 5, 20 μM), γ CEHC (1, 5, 20 μM) and trolox (20 μM) are added to various samples of anti-coagulated blood 10 minutes before the start of an experiment. In each experiment, blood is perfused for 5 minutes.

αCEHC, γ CEHC and trolox (20 μM) are a gift from Dr. Kenneth Hensley, OMRF. All the other chemicals are obtained from Sigma chemicals (MO, U.S.A) unless otherwise stated.
Protein Coating of Cover Slips

Glass cover slips (24x50 mm) (Fisher Scientific) are placed in 95% ethanol bath for at least 12 hours. Before use, each cover slip is rinsed with 95% ethanol and allowed to air-dry for 15 minutes.

Collagen type I solution is prepared at 0.8 mg/mL in 17 mM acetic acid (pH 2.6) at least 24 hours prior to use. Bovine serum albumin solution is prepared at 0.1% in 10 mM HEPES/115 mM NaCl buffer (pH 7.4). Half of each cover slip is coated with collagen and allowed to incubate for 4 hours in a humidified environment (80-90%) at room temperature (~24 °C). After incubation, each cover slip is rinsed with 10-15 mL of HEPES/NaCl buffer solution to remove excess collagen and is immersed in 0.1% albumin solution for at least 1 hour to coat the remaining part of cover slip.

Image analysis

Blood is allowed to flow on the cover slip coated with collagen using a syringe pump. Syringe pump provides the required flow rate of blood through the flow chamber. The coverslip is placed on the flow chamber, which is mounted on the stage of a Nikon Diaphot 300 inverted microscope. The fluorofor mepacrine is excited by a 75W Xenon light source and platelets are viewed through a 40 × fluorite objective lens. The fluorescent emission from adherent platelets is passed to an image intensifier and CCD camera and is recorded on VHS tape. The images from the tapes are captured by Dell workstation running ISEE® image analysis software by Inovision. The extent of thrombus formation is quantified by measuring the % of collagen surface covered by platelets at collagen-albumin interface. A background image is obtained by recording the flow on albumin surface, where there is no adhesion of platelets. This image is subtracted from the actual collagen images to reduce the noise in the images. The size and intensity parameters are adjusted to obtain the % coverage of platelets on collagen surface. Experiments are performed for 5 minutes and images are acquired every 30 seconds.

Aggregometry studies

Blood is drawn into BD 20 ml syringes with 0.9 % sodium citrate as anti coagulant. Platelet rich plasma (PRP) is obtained by centrifuging whole blood at 800 RPM for 10 min. The platelet count for all experiments is between 300 – 400 * 10^6 platelets / ml. Platelet poor plasma (PPP) is obtained by centrifuging PRP at 10,000 RPM for 5 min. Platelets are then incubated under various conditions, as mentioned above, with continuous stirring at 37°C. A PAP- 4 platelet aggregometer (Bio data corp, PA) is used for the aggregometer studies. Baseline for platelet aggregation is set by using PPP (200 µl). Convulxin (20 µg/ml) (generous gift from Dr. George Dale, OUHSc) is used for platelet aggregation and % of aggregation per minute is recorded on the aggregometer chart.

In all studies, blood is used in experiments within two hours of collection. Blood from three different donors is used and each experiment is done in triplicates. The order of experiments is varied randomly to insure that time-dependent platelet activation is not skewing the results.
Statistical Analysis

Results are expressed as mean±SEM. Statistical significance is determined using ANOVA. Significance is assumed if p<.05, otherwise the two samples are not considered to be significantly different. All statistical tests are performed using Excel.

Results and Discussion

γT but not αT decreases the rate of platelet adhesion and aggregation

Blood is perfused in the flow chamber for 5 minutes and % platelet coverage is quantified at every 30 seconds of the experiment. The effects of αT and γT on platelet adhesion and aggregation are studied at shear rates of 1000 S⁻¹ (Figure 2A) and 100 S⁻¹ (Figure 2B) of blood perfusion. At arterial shear rate of 1000 S⁻¹, blood incubated with γT 50μM has shown increased inhibition of platelet adhesion to collagen surface whereas αT (50μM) showed no effect on platelet aggregation (Figure 2 A). Decrease in % platelet coverage on collagen surface was significant (p< 0.05) up to 5 min with γT (50μM) and αT (50μM). At a shear rate of 100 s⁻¹ neither αT nor γT have any effect on platelet aggregation (Figure 2B). It has been shown that at low shear rates, apart from collagen receptors, a number of different adhesion molecules some of which do not require platelet activation are involved in thrombus formation. At arterial shear rates (1000 s⁻¹), platelet integrins are shown to play more important role in thrombus formation.
Carboxy ethyl hydroxyl chromans (CEHCs) reduce platelet activation at high shear

At a shear rate of 1000 s⁻¹, αCEHC and γCEHC (5μM) have significant effect on inhibiting platelet aggregation (Figure 2C). However, CEHCs have no effect on platelet aggregation at 100 s⁻¹ (Figure 2D).

CEHCs increase platelet inhibition partly by NO production

In order to understand the mechanism by which tocopherols and CEHCs affect platelet adhesion and aggregation, blood is perfused in the flow chamber in the presence of agonist (tocopherols or CEHCs) and L-NAME (L-Nitro arginine methyl ester (200 nM)), a potent inhibitor of NO production. Results are shown in figure 3A and 3B for the shear of 1000 s⁻¹. In the presence of L-NAME and tocopherols, the % platelet coverage after 1 min of blood perfusion remains same as in the presence of tocopherols alone indicating that NO does not play a role in inhibiting platelet adhesion in the presence of tocopherols. In the presence of L-NAME and CEHCs, the % platelet coverage after 1 min of blood perfusion has increased significantly (p<0.05) when compared with that of CEHCs alone but not as much as control indicating that the effect of CEHCs on inhibiting platelet adhesion and aggregation can be partly attributed to the production of NO by platelets in the presence of CEHCs.

Dose dependent effect of tocopherols and CEHCs on platelet adhesion

Different concentrations of tocopherols (20, 50 μM) are tested to see the dose dependent effect on initial stages of platelet aggregation. % platelet coverage after 1 min of blood perfusion vs. concentration curves showed that γT alone has a dose dependent effect on platelet aggregation (Figure 4A). Different concentrations of CEHCs (1, 5 and 20 μM) are tested to see the dose dependent
effect on initial stages of platelet aggregation. % platelet coverage after 1 min of blood perfusion vs. concentration curves showed that both CEHCs have dose dependent effect (Figure 4B). When 20 μM of CEHCs is used, there is no platelet adhesion on the collagen surface.

Platelet aggregometer studies

Platelet aggregometer studies showed that the αT, γT and CEHCs have effect on platelet aggregation induced by convulxin (which is an analogue of collagen) (Figure 5). These aggregometry studies show that tocopherols and CEHCS have similar effect on platelet aggregation indicating that these drugs have significant effect on collagen receptors.

![Figure 5: Effect of tocopherols (50 μM) and CEHCs (5 μM) on platelet aggregation in a platelet aggregometer. (*, #, p < 0.05)](image)

4. Discussion

We have seen that αT did not have any effect on reducing the platelet aggregation whereas γT and other metabolites of tocopherols have significant effect on inhibiting platelet adhesion and aggregation on collagen surface. The quinoline form of CEHCs, trolox has anti-inflammatory properties but showed no effect in inhibiting platelet aggregation, which leads us to assume that the anti-aggregating properties of tocopherols and CEHCs can be attributed to their methyl group substitutions on the chromanol ring structure and the phytal tails. The small size of the metabolites is assumed to have advantage over αT and γT, as they can easily reach the cells in solution. Recent studies have shown that the concentration of gamma tocopherol is less in the patients with cardiovascular diseases than in healthy population. Our study is the first one to present the direct effect of tocopherols and their metabolites on platelets.

These results led us carry further research on platelet – tocopherol interactions and signaling mechanism. As first step towards this goal, we assumed that tocopherols bind to peroxisome proliferator-activated - gamma receptor (PPAR - γ), a nuclear receptor which is recently shown to be present in platelets (since the structures of tocopherols and TZDs are similar and TZDs are shown to be PPAR – γ ligands)\(^7,8,19\).

References


Session 1: Oral Presentations (A235, Sarkeys Energy Center)
8:25  Welcome (Roger Harrison)
8:30  Engineering an aerobic homoacetogenic pathway in E. coli, Kevin Smith, Youyoun Moon, and Ramon Gonzales, Iowa State University
8:50  Design of a animal waste treatment system by combination of technologies, Sigifredo Castro, Larry Erickson, Alok Bhandari, Larry Davis, and Dean Thomson, Kansas State University
9:10  Supercritical carbon dioxide treatment of lignocellulosic biomass to enhance cellulose hydrolysis, Muralidhar Mallem and Patrick Gilcrease, South Dakota School of Mines and Technology
9:30  Glucose biosensor based on novel ferrocene poly(ethyleneimine) redox polymer, Steve Merchant and David Schmidlke, University of Oklahoma
9:50  Recovery and purification of recombinant protein from transgenic corn by aqueous two-phase partitioning, Zhengrong Gu and Charles Glatz, Iowa State University

Break
10:10-10:25

Session 2: Oral Presentations (A235, Sarkeys Energy Center)
10:25  Enzymatic esterification of geraniol in ionic liquid [bmim]PF₆ mediated by pervaporation, Donifan Barahona, Mary Rezac, and Peter Pfromm, Kansas State University
10:45  Characterization of polished dental materials by atomic force microscopy, Jacob Hedden, J. Lindblom, S. Sautter, Edgar O'Rear, and Sharukh Khajotia, University of Oklahoma
11:05  A novel class of polyanhydrides with tailored erosion mechanisms, Maria Torres, Balaji Narasimhan, and Surya Mallapragada, Iowa State University
11:25  Oxygen toxicity to bifidobacteria, Amit Apte, D.Y.C. Fung, and Larry Erickson, Kansas State University
11:45  Targeting L-methioninase to human cancer cells, Naveen Palwai and Roger Harrison, University of Oklahoma

Lunch
12:05-1:30

Session 3: Poster Presentations (West Atrium, First Floor, Sarkeys Energy Center)
1:30-2:15
1. Membrane coalescence of soy oil emulsions formed during aqueous oil extraction, Cheng Zhang and Charles Glatz, Iowa State University
2. Exploration of family 1 carbohydrate binding module structure and function by automated docking, Chandrika Mulakala, and Peter Reilly, Iowa State University
3. The environmental knowledge and assessment of tool (EKAT), Amit Apte, Edward D'Souza, Terrie Boguski, Ryan Green, and Larry Erickson, Kansas State University
4. In-situ biostimulation for remediation of chlorinated solvents in groundwater: a laboratory study, S. Santharam, L.C. Davis, and L.E. Erickson, Kansas State University
5. Microcosm investigation of the dechlorination of tetrachloroethene, J. Ibbini, L.C. Davis, and L.E. Erickson, Kansas State University
6. Protein separation using isoelectric focusing and field gradient focusing, E. D'Souza, C.T. Culbertson, and L.E. Erickson, Kansas State University
7. Preliminary studies on the deoxygenation of fatty acids in hydrocarbon feedstocks, Amal More, John Schlup, and Keith Holn, Kansas State University
8. Improving ethanol yields from lignocellulosic biomass using a novel pretreatment, Katherine Standish, Muralidhar Mallem, and Patrick Gilcrease, South Dakota School of Mines and Technology
9. Enhancing biological methane production from biomass, Michael Green and Patrick Gilcrease, South Dakota School of Mines and Technology
10. Influence of spatial architecture on cell shape and colonization on polysaccharide based scaffolds, Yan Huang, Mbonda Siewe, and Sundararajan Madihally, Oklahoma State University
11. Auto-dissection and cell-hydrogel loading of ex vivo scaffolds for vascular reconstructive surgery, Joel Daniel, Koki Abe, and Peter McFetridge, University of Oklahoma
12. Chemical modification of single-walled nanotubes (SWNT) improves in vitro cell-SWNT interactions, Aditya Nimmagadda, Karen Thurston, Matthias Nollert, and Peter McFetridge, University of Oklahoma
13. Preparation of a flexible, biometric 3D scaffold for tissue engineering applications, Jose Alvarez, Paul DeAngelis, Jessica Yankovich, Mark Shreve, and Vassilios Sikavitsas, University of Oklahoma
14. Fabrication of protein patterned surfaces via particle lithography, Krupa Patel, Andrew Carswell, and David Schmidtke, University of Oklahoma
15. Glucose biosensors based on layer-by-layer redox polymer-enzyme-carbon nanotube films, Youdan Wang, Pratixa Joshi, and David Schmidtke

Session 4: Oral Presentations (A235, Sarkeys Energy Center)
2:15 Effect of blending with polycaprolactone on the anti-bacterial activity of chitosan for periodontal tissue engineering, Apama Sarasam, John Dmytryk, Sharukh Khajotia, and Sundararajan Madihally, Oklahoma State University
2:35 Flow perfusion improves seeding efficiency in scaffolds for bone tissue engineering, Jose Alvarez-Barreto, Robert Shambaugh, S. Linehan, Jessica Yankovich, and Vassilios Sikavitsas, University of Oklahoma
2:55 Design of polyanhydride microspheres as protein carriers, Jennifer Graham, Katie Pfeiffer, Amy Determan, and Balaji Narasimhan, Iowa State University
3:15 Tocopherols and CEHCs modulate platelet thrombus formation, Durga Sarvepalli, Ken Hensley, and Ulli Nollert, University of Oklahoma

Invitation to Attend the 2006 Symposium
3:35