Proceedings of the 39th Annual Biochemical Engineering Symposium

Larry E. Erickson
Kansas State University

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Proceedings of the 39th Annual Biochemical Engineering Symposium

Kansas State University

Larry E. Erickson
Editor

April 17, 2010

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

The 39th Annual Biochemical Engineering Symposium was held at Kansas State University on Saturday, April 17, 2010. The conference provides an opportunity for students to present their research to faculty and students from the participating universities and to discuss their research with interested individuals. Thirty-five individuals participated in the symposium.

This proceedings includes manuscripts of several of the presentations and abstracts of many of the other presentations. Readers who want more information on a topic are encouraged to contact the authors directly. Some of the research has been submitted to journals that publish papers after peer review. The program and a listing of the participants are included.

The editor would like to thank all of the authors and presenters for their participation in the symposium, and Florence Sperman for helping to prepare the manuscripts for publication in this proceedings. Financial support from the Kansas State University Department of Chemical Engineering and the Center for Hazardous Substance Research is gratefully acknowledged.

Larry E. Erickson
Editor
February 25, 2011

LIST OF PARTICIPANTS

Colorado State University
Laura Place and Emily Stump

Iowa State University
Zachary Beversdorf, David Cantu, Yingfei Chen, Tingsong Dai, You Hou, Xunpei Liu, Peter Reilly, Ryan Swanson

Kansas State University

University of Oklahoma
Selda Goktas, Roger Harrison, Luis Neves, Hoai Nguyen, Samuel B. Van Gordon, Brent D. Van Rite
Oral Presentation Schedule

8:30 Ultrafast, Label-free Detection of Pathogenic Bacteria via Peptide-modified-Graphene Biosensor
Nihar Mohanty, Angela D. Adams, Duy H. Hua, and Vikas Berry

8:55 Classification of Ketoacyl Synthases by Their Primary and Tertiary Structures
Yingfei Chen and Peter J. Reilly

9:20 Scaffold Architecture's Effect on Preosteoblastic Cultures Exposed to Continuous Fluid Shear
Samuel B. VanGordon, Roman S. Voronov, Taren B. Blue, Robert L. Shambaugh, Dimitrios V. Papavassiliou and Vassilios I. Sikavitsas

9:45 Break for Poster Viewing

10:15 Production of Nitrogen-Based Platform Chemical: Cyanophycin Biosynthesis using Recombinant Escherichia coli
Yixing Zhang, Amit Kumar, Praveen Vadlani, and Sanjeev Narayanan

Nihar Mohanty, Ashvin Nagaraja, Monica Frey, and Vikas Berry

11:05 Proteomics Approach for Predicting Retention Behavior of a Mixture of Proteins during Hydrophobic Interaction Chromatography
Ryan Swanson and Charles E. Glatz

11:30 Break for Lunch and Poster Viewing

1:00 Designing Virus Surrogates
Emily Stump, Hailey Cutler, Guadalupe D. Gutierrez, Scott Husson, John Pellegrino, and Ranil Wichramasinghe

1:25 Evaluation of Different Agricultural Feedstocks for Bioethanol Production
Sunil Bansal, Praveen Vadlani, and Scott Staggenborg

1:50 Markovian Modeling and Monte Carlo Simulation of Bacterial Disinfection: Non-Linear Approach
Andres Argoti, L.T. Fan, and S.T. Chou

2:15 Break for Poster Viewing

2:45 The Use of Targeted Enzyme/Prodrugs for Treatment of Solid Tumors
Brent D. Van Rite, Magali Pagnon, Yahya Lazrak, and Roger G. Harrison

3:10 Biochemical Functionalization of Graphene for Cancer Cell Detection
Kabeer Jasuja and Vikas Berry

3:35 Conclusion of Symposium
Posters

1. **Highly Porous Uniform Pore Size Membranes for Ultrafiltration**  
   Xinying Wang, Scott Husson, Xianghong Qian, and Ranil Wickramasinghe

2. **Molecular Dynamics Investigation of the Effects of Ionic Strength on LCST of Poly(N-isopropylacrylamide)**  
   Hongbo Du, Ranil Wickramasinghe, and Xianghong Qian

3. **Bioinspired Synthesis of Calcium Phosphate Nanocomposites Templated by Block Copolymer-peptide Conjugates**  
   Xunpei Liu, Mathumai Kanapathipillai, Yusuf Yusufoglu, Yan-yan Hu, Mufit Akinc, Klaus Schmidt-Rohr and Surya Mallapragada

4. **Regulation of Focal Adhesion Maturation and Cell Edge Dynamics by Epidermal Growth Factor**  
   Yue Hou and Ian Schneider

5. **Development of a Bioreactor system for the Cultivation of an Engineered Periodontal Graft**  
   Selda Goktas, John J. Dmytryk and Peter S. McFetridge

6. **Targeted Single-Walled Carbon Nanotubes for the Treatment of Cancer**  
   Luis F.F. Neves, Whitney Prickett, Daniel E. Resasco, and Roger G. Harrison

7. **Development of Fast-acting Microspheres for Thrombolytic Therapy**  
   Hoai Nguyen, and Edgar O'Rear, and Eugene Patterson

8. **Production and Characterization of Porins**  
   Sebastian Wendel, Stefan Bossmann, Peter Pfromm, and Peter Czermak

9. **Field Investigation of the Bioremediation of Chlorinated Ethenes in Groundwater**  
   Mark A. McClure, L.C. Davis, and L.E. Erickson

10. **Growth of Algae Using Nitrogen and Phosphorus in Wastewater**  
    Richard Reed, Larry E. Erickson and Wenqiao Yuan

11. **Catalytic Domain Organization in Hydroxyacyl Dehydratases, Enoyl Reductases, and Ketoacyl Reductases**  
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Sebastian Wendel, Stefan Bossmann, Peter Pfommm, and Peter Czermak, “Production and Characterization of Porins”, KSU
Classification of ketoacyl synthases by their primary and tertiary structures

Yingfei Chen and Peter J. Reilly

Department of Chemical and Biological Engineering, Iowa State University, Ames, Iowa 50011

Abstract: Ketoacyl synthases (KSs), encompassing 20 entries in the EC 2.3.1 classification, catalyze fatty acid elongating reactions combining acyl-CoA or acyl-ACP with malonyl-CoA or malonyl-ACP to form 3-ketoacyl-CoA or 3-ketoacyl-ACP. They also elongate other activated molecules as part of the polyketide synthesis cycle. Our classification of KSs by their primary and tertiary structures instead of by their substrates and the reactions that they catalyze provides some insights into this enzyme group. At present, KSs fall into five families whose members have the same catalytic residues, mechanisms, and tertiary structures. Four families are in one clan, where they share similar tertiary structures and mechanisms but have less related primary structures. KS sequences, tertiary structures, and family classifications are available on the continuously updated ThYme (Thioester-active Enzyme) database.

Introduction

Ketoacyl synthases (KSs) are the condensing enzymes that catalyze the reaction of acyl-CoA or acyl-ACP with malonyl-CoA, malonyl-acyl carrier protein (malonyl-ACP), or occasionally other substrates. This reaction is a key step in the fatty acid elongation cycle, as in general it adds two carbon atoms to the acyl chain (Figure 1). KSs exist as individual enzymes; furthermore, KS domains are found in multi-modular enzymes such as fatty acid synthase (FAS) and polyketide synthase (PKS) (Smith et al., 2007).

We have gathered KS amino acid sequences (primary structures) and three-dimensional (tertiary) structures, along with those of other members of the fatty acid elongation cycle, into the continually updated ThYme (thioester-active enzYme) database (www.enzyme.cbirc.iastate.edu) (Cantu et al., 2010, 2011). In doing this, we divided each of these enzyme groups into different families based on their primary structure differences. In general, single families contain enzyme members that are related to each other by primary and tertiary structure and mechanism, suggesting that they have a common ancestor, while those in different families are only slightly or not at all related, suggesting that they are descended from different ancestors. Sometimes members of different families are sufficiently related by primary and tertiary structures and by mechanism that they can be classified as part of a clan, implying that they are descended from a more distant common ancestor. Furthermore, we can divide members of a single family into subfamilies by more subtle primary structural differences.

This article is an account of our division of KSs into families, the gathering of some of the families into clans, and the separation of families into subfamilies. We have done this so that, with the help of known KS crystal structures, mechanisms, and substrate specificities, we could rationally predict the properties of KSs according to the phylogenetic trees that we constructed.

Three small-scale phylogenetic trees of KSs have already been built. An early tree based on seven tertiary structures showed that ketoacyl-ACP synthase II from the cyanobacterium Synechocystis and Escherichia coli ketoacyl-ACP synthases I and II were very similar, as were Saccharomyces cerevisiae degradative thiolase and Zoogloea ramigera biosynthetic thiolase.
More distant from each other and from the other two groups were alfalfa chalcone synthase and *E. coli* ketoacyl-ACP synthase III (Moche et al., 2001). A phylogenetic tree of 18 known *Arabidopsis* 3-ketoacyl-CoA synthases and putative genes has been produced to identify these enzymes in putative enzymes (Blacklock and Jaworsky, 2006). A phylogenetic study of 40 β-ketoacyl-acyl carrier protein synthase III enzymes showed that those produced by bacteria (proteobacteria, firmicutes, and bacteroidetes) and an apicomplexans protist species are widely separated from those produced by monocots, dicots, diatoms, cyanobacteria, and red and green algae (González-Mellado et al., 2010).

**Family identification**

Basic Local Alignment Search Tool (BLAST) (Altschul et al., 1997) and multiple sequence alignment (MSA) were used to classify KSs into different families based on primary structure similarity, while crystal structure superposition and root mean square (RMSD) calculation were used on tertiary structures. More complete descriptions of these methods are found in the Supporting Information of an earlier article (Cantu et al., 2010).

The query sequences for BLAST were all the KSs with evidence at protein level, ensuring that they all had associated experimental data. Twenty of 187 entries in Enzyme Commission (EC) 2.3.1 (NC-IUBMB, 1992) are KSs, but four of them have no sequences with evidence at protein level, leaving query sequences to be retrieved from the UniProt database (UniProt Consortium, 2008) for the remaining 16 EC numbers. Only the KS catalytic domain of each enzyme, obtained from the Pfam database (Sonnhammer et al., 1997), was used. If no Pfam entry appeared, then the hidden Markov model by HMMER 3.0 (Eddy, 1998) was employed to find the KS catalytic domain.

The Linux form of BLAST (version 2.2.19) was used to find enzymes with similar primary structures in the Non-Redundant (NR) protein sequence database, based on an E-value of 0.001 and a maximal target sequence value of 100,000. A script was written to run BLAST successively and to classify KSs into potential families.

MSA with MUSCLE 3.6 (Edgar, 2004) and ClustalX 2.0.10 (Larkin et al., 2007) using default parameters was conducted on a sample of approximately 50 sequences from each potential family or between different potential families, to determine whether the former should be split into two or more families or whether the latter should be merged into fewer families.

The KS catalytic domains of all KS crystal structures in each family obtained from the RCSB Protein Data Bank (www.pdb.org) were superimposed in MultiProt (Shatsky et al., 2004). Then, a MATLAB (http://www.mathworks.com/matlabcentral/) script was used to calculate the RMSD of the distance of each α-carbon atom between different tertiary structures (Cantu et al., 2010). Since the tertiary structures of different enzymes may be of different sizes, their numbers of α-carbon atoms may differ. Therefore $P_{av}$ values, indicating the average percentage of α-carbon atoms compared, was also recorded. Furthermore, tertiary structures superimposed by PyMOL (http://www.pymol.org/) were visually checked.

**Ketoacyl synthase families**

Based on these techniques, KSs are divided into five families, totaling at present over 17,600 sequences (Table I).

Most KS1 members are produced by bacteria, with some formed by eukaryota and only one from an archaeon. The dominant enzyme in this family is 3-ketoacyl-ACP synthase III, also called β-ketoacyl-ACP synthase III, and the dominant EC numbers are EC 2.3.1.41 and EC
2.3.1.180. The most common reactions catalyzed by KS1 enzymes are malonyl-ACP with acyl-CoA or acyl-ACP to form a 3-ketoacyl-ACP molecule two carbon atoms longer than before, along with CO₂ and CoA or ACP (Figure 1).

All KS2 enzymes are from eukaryota, with nearly all from plants. 3-Ketoacyl-CoA synthase, fatty acid elongase, and very long-chain fatty acid condensing enzyme are the most common enzymes in this family. Some are defined as EC 2.3.1.119, but the general characterization as EC 2.3.1.– is more common.

KS3 is the largest KS family, containing at present over 11,300 sequences. KSs in this family include the KS domains of large multimodular enzymes such as fatty acid synthases (FAsS) and polyketide synthases (PKSs). Many different enzymes are included in this family, but the largest number of members are 3-ketoacyl-ACP synthases I and II and undifferentiated PKSs, with EC 2.3.1.41 and EC 2.3.1.179 as the most common EC numbers. Bacteria produce most KS3 members; eukaryota are substantial producers, and a few KS3 enzymes are of archaeal origin.

A large fraction of KS4 enzymes are from eukaryota, while the remaining ones are from bacteria. They are classified as chalcone synthases, stilbene synthases, and naringenin-chalcone synthases, and overwhelmingly those that have EC numbers are listed as EC 2.3.1.74.

KS5 members are all from eukaryota, and most are produced by animals. They are mainly fatty acid elongases and elongation of very long chain fatty acid proteins. At present none has an EC number.

Each family appears to have members that are specific for either malonyl-ACP or malonyl-CoA and for either an acyl-ACP or an acyl-CoA, indicating that specificity for activating groups is not a criterion for family membership. This differs from that found with thioesterase families (Cantu et al., 2010).

Although KSs of various types have 20 EC entries, only five comprise the great majority of enzymes gathered by using BLAST with query sequences taken from enzymes with evidence at protein level (Table I). These numbers, assigned by groups working on KSs, are EC 2.3.1.41 (β-ketoacyl-ACP synthase I), EC 2.3.1.74 (naringenin-chalcone synthase), EC 2.3.1.119 (icosanoyl-CoA synthase), EC 2.3.1.179 (β-ketoacyl-ACP synthase II), and EC 2.3.1.180 (β-ketoacyl-ACP synthase III) (NC-IUBMB, 1992). The reactions that they characteristically catalyze are shown in Table II. These factors suggest that KS1 contains enzymes that catalyze elongating reactions specific to short acyl chain lengths, while enzymes in KS2, KS3, and KS5 elongate longer acyl chains, and KS4 enzymes specifically produce chalcones.

**Overlapping sequences in different families**
Some amino acid sequences have been assigned to more than one KS family by use of BLAST with query sequences gathered as described above. KS1, with 2308 sequences, and KS2, with 375 sequences, have 23 in common. KS1 and KS4 (1229 sequences) have 92 in common. KS2 and KS4 share 72 sequences. The overlapped sequences were placed into KS families according to the enzyme names assigned by the researchers who obtained them. We kept KS1, KS2, and KS4 as separate families because an MSA shows clearly that sequences in each family are substantially different from sequences in the other two families (Supporting Information). Furthermore, the enzymes in these three families have rather different reported specificities and producing organisms (Tables I and II).

**Ketoacyl synthase crystal structures**
All known tertiary structures of members of KS1, KS3, and KS4 have thiolase-like folds, with
five layers of α-β-α-β-α structure (Huang et al., 1998). KS2 and KS5 have no crystal structures. KS1 has 37 crystal structures, with an RMSD_{ave} values obtained by superposition of these structures of 1.25 Å and a P_{ave} value of 94.7%. The corresponding values for KS3 are 63 structures, 1.46 Å, and 68.3%, and those for KS4 are 38 structures, 0.89 Å, and 95.8%.

**Ketoacyl synthase catalytic residues and mechanisms**

Based on crystal structures and consistent with previous results with thioesterases (Cantu et al., 2010), catalytic residues are well conserved within KS1, KS3, and KS4 (Table III). This leads us to assume that all members of a family will have the same mechanism, which with them is the ping-pong kinetic mechanism (Plowman, 1972), using cysteine, histidine, and either histidine or asparagine as a catalytic triad.

Cysteine, histidine, and asparagine form the catalytic triad in KS1. Qiu et al. (1999) proposed that Cys112 in *E. coli* β-ketoacyl-ACP synthase III (PDB entry 1HN9) donates a proton to His244 and attacks acetyl-CoA. Then malonyl-ACP is attached to His244 and Asn274 to be decarboxylated, forming a carbanion. Finally, the carbanion attacks the acetyl moiety to form acetoacetyl-ACP.

In KS2, mutagenetic analysis of *Arabidopsis* FAE1 β-ketoacyl-CoA synthase strongly suggested that it shares the same ping-pong mechanism and putative Cys-His-Asn/His catalytic residues as members of KS1, KS3, and KS4 (Ghanevati and Jaworski, 2002).

The KS3 active site has a Cys-His-His triad. In *Streptococcus pneumoniae* KASII (2ALM), acyl-ACP transfers its acyl moiety to Cys164. Then, it is proposed that a water molecule activated by His303 attacks malonyl-ACP to form a carbanion. His337 also stabilizes the malonyl moiety. Last, the carbanion attacks the acyl moiety and forms β-ketoacyl-ACP (Zhang et al., 2006).

KS4 has a Cys-His-Asn catalytic triad, the same as KS1. The chalcone synthase/stilbene synthase superfamily catalyzes, beside the same acyl transfer, decarboxylation, and condensation steps as KS1, further cyclization and aromatization before it forms the final chalcone product (Austin and Noel, 2002).

Little is yet known about the catalytic mechanism of KS5.

**Ketoacyl synthase clans**

Although amino acid sequences of members of different families may completely or almost completely differ from each other, their crystal structures, catalytic residues, and mechanisms may be conserved, indicating that they have a distant common ancestor, and therefore are part of the same clan.

Crystal structures from KS1, KS3, and KS4 were superimposed and their RMSDs were determined to see whether these families comprised a single clan. The RMSD of the superimposed structures of KS1, KS3, and KS4 (Figure 2), one from each family, is 1.96 Å, with a P_{ave} of 68.5%. Furthermore, the catalytic residues of members of the three families occupy the same positions (Figure 3), and their secondary structure elements are found in the same order (Table IV) Thus they fall into one clan. Considering that KS2 has so many sequences that overlap with sequences in KS1 and KS4, this family is undoubtedly also part of the same clan.

**References**


### Table I. Ketoacyl Synthase Families and Common Names of their Members

<table>
<thead>
<tr>
<th>Family Producing organisms</th>
<th>Dominant EC numbers</th>
<th>Dominant enzyme names</th>
</tr>
</thead>
<tbody>
<tr>
<td>KS1 A, B, E</td>
<td>2.3.1.41, 2.3.1.180</td>
<td>3-Ketoacyl-ACP synthase III</td>
</tr>
<tr>
<td>KS2 E</td>
<td>2.3.1.119, 2.3.1.180</td>
<td>3-Ketoacyl-CoA synthase, fatty acid elongase, very long-chain fatty acid</td>
</tr>
<tr>
<td>KS3 A, B, E</td>
<td>2.3.1.41, 2.3.1.179</td>
<td>3-Ketoacyl-ACP synthase I and II, KS domain of FAS or PKS</td>
</tr>
<tr>
<td>KS4 B, E</td>
<td>2.3.1.74</td>
<td>Chalcone synthase, stilbene synthase, naringenin-chalcone synthase</td>
</tr>
<tr>
<td>KS5 E</td>
<td></td>
<td>Elongation of very long chain fatty acid protein, fatty acid elongase</td>
</tr>
</tbody>
</table>

* A, archaea; B, bacteria; E, eukaryota. Most prevalent producers bolded.

### Table II. Ketoacyl Synthases Commonly Found in Thyme

<table>
<thead>
<tr>
<th>EC number</th>
<th>Enzyme name</th>
<th>Catalyzed reaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.3.1.41</td>
<td>β-Ketoacyl-ACP synthase I</td>
<td>Malonyl-ACP + acyl-ACP → 3-ketoacyl-ACP + CO₂ + ACP</td>
</tr>
<tr>
<td>2.3.1.74</td>
<td>Naringenin-chalcone synthase</td>
<td>3 Malonyl-CoA + 4-coumaroyl-CoA → naringenin chalcone + 3 CO₂ + 4 CoA</td>
</tr>
<tr>
<td>2.3.1.119</td>
<td>Icosanoyl-CoA synthase + CO₂ + CoA + H₂O</td>
<td>Malonyl-CoA + stearoyl-CoA + 2 NAD(P)H + 2 H⁺ → icosanoyl-CoA + 2 NAD(P)⁺</td>
</tr>
<tr>
<td>2.3.1.179</td>
<td>β-Ketoacyl-ACP synthase II</td>
<td>Malonyl-ACP + (Z)-hexadec-11-enoyl-ACP → (Z)-3-oxoocadeca-13-enoyl-ACP</td>
</tr>
<tr>
<td>2.3.1.180</td>
<td>β-Ketoacyl-ACP synthase III</td>
<td>Malonyl-ACP + acetyl-CoA → acetoacetyl-ACP + CO₂ + CoA</td>
</tr>
</tbody>
</table>
Table III. Catalytic Residues of KS Families

<table>
<thead>
<tr>
<th>Family</th>
<th>Producing organism</th>
<th>Gene</th>
<th>Catalytic residues&lt;sup&gt;a&lt;/sup&gt;</th>
<th>PDB file</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td><em>Escherichia coli</em></td>
<td>FabH</td>
<td>Cys112, His244, Asn274</td>
<td>1EBL, 1HND, 1HNH, 1HNJ, 1HNK</td>
</tr>
<tr>
<td>1</td>
<td><em>Pseudomonas aeruginosa</em></td>
<td>PqsD</td>
<td>Cys112, His257, Asn287</td>
<td>3H76, 3H77, 3H78</td>
</tr>
<tr>
<td>1</td>
<td><em>Staphylococcus aureus</em></td>
<td>FabH</td>
<td>Cys112, His238, Asn268</td>
<td>1ZOW</td>
</tr>
<tr>
<td>3</td>
<td><em>E. coli</em></td>
<td>FabB</td>
<td>Cys163, His298, His333</td>
<td>1FJ4, 1FJ8</td>
</tr>
<tr>
<td>3</td>
<td><em>Homo sapiens</em></td>
<td>OXSM</td>
<td>Cys209, His348, His385</td>
<td>2IWY, 2IWZ</td>
</tr>
<tr>
<td>3</td>
<td><em>Mycobacterium tuberculosis</em></td>
<td>KasB</td>
<td>Cys170, His311, His346</td>
<td>2GP6</td>
</tr>
<tr>
<td>3</td>
<td><em>M. tuberculosis</em></td>
<td>KasA</td>
<td>Cys171, His311, His345</td>
<td>2WGD, 2WGE, 2WGF, 2WGG</td>
</tr>
<tr>
<td>3</td>
<td><em>Saccharomyces cerevisiae</em></td>
<td>FAS2</td>
<td>Cys1305, His1542, His1583</td>
<td>2PFF, 2UV8</td>
</tr>
<tr>
<td>3</td>
<td><em>Saccharopolyspora erythraea</em></td>
<td>EryA</td>
<td>Cys199</td>
<td>2HG4</td>
</tr>
<tr>
<td>3</td>
<td><em>S. erythraea</em></td>
<td>EryA</td>
<td>Cys202, His337, His377</td>
<td>2QO3</td>
</tr>
<tr>
<td>3</td>
<td><em>Streptococcus pneumoniae</em></td>
<td>FabF</td>
<td>Cys164, His303, His337</td>
<td>1OX0, 1OXH, 2ALM</td>
</tr>
<tr>
<td>3</td>
<td><em>Thermus thermophilus</em></td>
<td>FabF</td>
<td>Cys161, His301, His338</td>
<td>1J3N</td>
</tr>
<tr>
<td>4</td>
<td><em>Aloe arborescens</em></td>
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<td>Cys174, His316, Asn349</td>
<td>2D3M, 2D51, 2D52</td>
</tr>
<tr>
<td>4</td>
<td><em>Arachis hypogaea</em></td>
<td>—</td>
<td>Cys164, His303, Asn338</td>
<td>1Z1E, 1Z1F</td>
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<tr>
<td>4</td>
<td><em>Medicago sativa</em></td>
<td>CHS2</td>
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<td>1JWX</td>
</tr>
<tr>
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<td>1TED, 1TEE</td>
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<tr>
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<td><em>Neurospora crassa</em></td>
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<td>—</td>
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<td>1U0U</td>
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<tr>
<td>4</td>
<td><em>Rheum palmatum</em></td>
<td>Bas</td>
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<td>3A5Q, 3A5R, 3A5S</td>
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<tr>
<td>4</td>
<td><em>Streptomyces coelicolor</em></td>
<td>—</td>
<td>Cys138, His270, Asn305</td>
<td>1U0M</td>
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</table>

<sup>a</sup> All the catalytic residues are gathered from literature associated with PDB structures.
Table IV. Secondary Structure Elements of Ketoacyl Synthase Families

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<tr>
<th>Family</th>
<th>Secondary Structural Element$^a$</th>
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</thead>
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<td>1 - $\beta$ $\alpha$ $\alpha$ $\beta$ $\alpha$ $\alpha$ $\beta$ $\alpha$ $\beta$ - $\alpha$ $\beta$ $\alpha$ $\beta$ - $\alpha$ $\beta$ $\alpha$ $\alpha$ - $\beta$ $\beta$</td>
<td></td>
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<td>3 - $\beta$ - $\alpha$ - $\alpha$ $\alpha$ $\beta$ $\alpha$ $\alpha$ $\beta$ $\alpha$ $\beta$ $\alpha$ $\alpha$ $\beta$ $\alpha$ $\beta$ $\alpha$ $\alpha$ $\beta$ $\alpha$</td>
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<tr>
<td>4 $\alpha$ $\beta$ $\alpha$ $\alpha$ $\beta$ $\alpha$ $\alpha$ $\beta$ $\alpha$ $\beta$ $\alpha$ $\alpha$ $\beta$ $\alpha$ $\beta$ $\alpha$ $\alpha$ $\beta$ $\alpha$</td>
<td></td>
</tr>
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</table>

$^a$ $\alpha$: $\alpha$-helix; $\beta$: $\beta$-sheet.

Figures

Figure 1. The fatty acid synthesis cycle.
**Figure 2.** Superimposed KS crystal structures. KS1 (yellow): 1EBL from *Escherichia coli* b-ketoacyl-ACP synthase III; KS3 (cyan): 2Q03 from *Saccharopolyspora erythraea* DEBS 2; KS4 (pink): 1Z1E from *Arachis hypogaea* stilbene synthase.

**Figure 3.** Superimposed KS active sites. KS1 (yellow): 1EBL from *Escherichia coli* b-ketoacyl-ACP synthase III; KS3 (cyan): 2Q03 from *Saccharopolyspora erythraea* DEBS 2; KS4 (pink): 1Z1E from *Arachis hypogaea* stilbene synthase. Bottom left: cysteine; bottom right: histidine; top: asparagine or histidine.
Growth of Algae using Nitrogen and Phosphorus in Waste Water

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¹Chemical Engineering, Kansas State University
²Biological and Agricultural Engineering
Kansas State University
Manhattan, Kansas 66506

Abstract

There are many drawbacks to the current fuel supply such as the depletion of oil, and the pollutants created upon using the fuel. These drawbacks to oil had led to interest into bio-fuels, those produced from biomaterial; however the some biomaterials are better than others. The goals of the project are to investigate the effect of wastewater concentration on the growth of Scenedesmus dimorphus (algae), to explore the growth of Scenedesmus dimorphus while being fed differing amounts of sodium bicarbonate daily, and to examine the effect of different photobio reactors (PBRs) on the growth of Scenedesmus dimorphus. In experiment one, five different media were used; 50% autoclaved wastewater, rest tap water preformed the best. For experiment two, sodium bicarbonate was tested as a fertilizer and was found to be effective in increasing the growth of the algae. Finally, the cylindrical PBRs were found more effective than the tubular PBRs.

Motivation

There are many drawbacks to the current fuel supply. There is an ever enlarging demand for oil. However, this very oil is in limited supply. Another problem with the traditional fossil fuels is the large amount of greenhouse gas emissions that are produced upon burning the fuels.

These drawbacks to oil have led to interest into bio-fuels, those produced from biomaterial. The main crop for bio-fuel is currently corn. This crop is easy to convert to fuel; however, there are many drawbacks to this source as well. A large cropping surface is needed to grow corn for fuel. Since corn is also a food product, this is a problem. In order to supply enough fuel to replace a meaningful amount of oil, a lot of crop land is taken up. It is impossible to sustainably use the land for both uses simultaneously.

This research project is exploring the use of algae as an alternative biofuel. There are many benefits for algae as a biomaterial. It uses a minimal amount of fertilizer, and it can be used to treat wastewater at the same time as growing it for fuel use. Algae uses water to grow in, thus it does not compete for land space, and it can be grown in places that do not have suitable cropland.
Research Objective

The goals of the current research are to investigate the effect of wastewater concentration on the growth of Scenedesmus dimorphus, to explore the growth of Scenedesmus dimorphus while being fed differing amounts of sodium bicarbonate daily, and to examine the effect of different photo-bio reactors (PBRs) on the growth of Scenedesmus dimorphus. This particular strain of algae was chosen because of the high lipid yield found for the strain. A high lipid yield would mean that the algae would produce the most bio-fuels as an end product, which is important in making the system more useful.

Figure 1 shows a map of the overall goal for the research. The thought is to take solid and liquid waste from an animal farm or feed lot and put it into an anaerobic digester. This would produce CO₂ and CH₄. The carbon dioxide from the digester would be bubbled through algae to use the CO₂ present. The resulting algae can be processed to remove carbohydrates and lipids that can be converted into bio-fuels. The remains of the algae can then be reloaded into the digester. The initial research will focus on growing algae most efficiently. This report is essentially presenting the research from the “Algae PBR System” block in Error! Reference source not found. Figure 1.

Summary of Previous Studies

Algae, specifically S. Dimorphus, are dependent on the amount of nitrogen and phosphorus in the culture medium (Kunikane, Kaneko, & Maehara, 1984). Each of the culture media utilized in the experiment started with differing amounts of the nitrogen and phosphorus, thus it was desirable to find the media that provided the most nutrients without becoming toxic for the algae. Another interesting study showed that sodium can supplement the phosphorus
requirement in plants (Allen & Arnon, 1955). They studied a different strain of algae, but it is expected that S. dimorphus will have a similar effect. Part of this report is on what effect the addition of sodium bicarbonate has on the growth of the algae. It was considered purely for the addition of dissolved carbon into the water, but it may have additional effects as well.

The technology of converting manure into biogas is essentially completed. The anaerobic digestion process is known and works well. A lot of study has gone into optimizing the amount of biogas released from the digester. Many waste utility plants that use anaerobic digestion as the method of waste treatment require almost no input energy to run. In fact, most produce excess energy. The mechanics of digestion are very well understood, but little study has been done linking anaerobic digestion to algae growth. Conventionally, anaerobic digestion produces methane and carbon dioxide where the CO₂ is currently being released into the atmosphere. When the digester is linked to an algae culture, the carbon dioxide can be converted into sugars and lipids by the plant mass (algae). The sugars can then be fermented to make bio-fuels.

In the digester a mixture of several types of bacteria including hydrolytic, acid forming, and methanogens work together to break down the complex organic matter and create methane and carbon dioxide (Hamilton, 2009). Past research has been done on what conditions provide the greatest volume of biogas. Wiegel (Ahring, 2003) found that biogas is produced best at temperatures between 55 and 65 degrees centigrade. Other studies have been done with respect to ammonia and other contaminates.

Materials and Methods

Experiment One

Five different media were used including: Basal, 50% Autoclaved wastewater (rest tap water), 100% autoclaved wastewater, 50% raw wastewater (rest tap water), and 100% raw wastewater. The only type of photo-bio reactor (PBR) used was the cylindrical type. Ten one liter graduated cylinders were used, thus two cylinders were used for each type of media. Each PBR was filled with 500 mL of the respective type of culture media and 100 mL of Scenedesmus dimorphus seed. Air pumps and tubes were used to bubble air through each cylinder to provide carbon dioxide to the cultures.

The experiment was to test what the best media was for growing Scenedesmus dimorphus. Raw wastewater was taken from a local source near Bill Snyder Family Stadium. Some of the wastewater was then autoclaved to kill off any other bacteria or algae in the water. For the 50% wastewater media, the rest was distilled water. Samples of the 100 percent raw and autoclaved wastewater were sent to the Kansas State University (KSU) Soils lab for analysis of nitrogen (N) and phosphorus (P). The results are shown in Table 1 of the appendix.

Every other day a ten milliliter sample was taken from each PBR and run through a filter paper that had been measured for weight. The resulting papers were left in an oven at 50 degrees centigrade overnight to dry the papers. The samples were weighed the next day and used to calculate the dry weight of algae in each PBR sample.
Experiment Two

It is well known that carbonate, bicarbonate, and carbon dioxide exist in equilibrium concentrations in solution. Using this fact, it was decided to try to increase the growth of algae by adding sodium bicarbonate to each PBR using the thought that it would increase the amount of dissolved CO₂, thus increase the algae growth. For the experiment sixteen PBRs were used, eight of each cylindrical and tubular. The only media used was 50% autoclaved wastewater because it performed the best in experiment one. The cylindrical PBRs were filled to 600 milliliters total while the tubular reactors were each filled to 1000 milliliters. The volume of algae seed was 7.58% of the liquid of each PBR. Halfway through the experiment, samples of each PBR were sent to the KSU Soils lab and the results of the water analysis are shown in Table 2. Finally, at the end of the testing period, samples were again sent to the Soils lab. The results are tabulated in Table 3.

The goal of this experiment was to see the difference between two types of PBRs and to test the effect of adding sodium bicarbonate (NaHCO₃) to the media, thus supplying CO₂ to the Scenedesmus dimorphus. NaHCO₃ was added every day in four different amounts: 0 g, 0.1 g, 0.5 g, and 1.0 g. Each feeding amount was repeated for each type of PBR thus using sixteen PBRs. The same method used in experiment one was used in experiment two to determine the concentration of algae in each PBR.

Experiment Three

This experiment was a small test performed at the beginning of experiment two to determine qualitatively if adding sodium bicarbonate to the photo-bio reactors would increase the amount of carbon dioxide dissolved in the solution. The set up was simple. Four flasks were set up with 200 mL of water. NaHCO₃ was added to each flask in the amounts used in one day of experiment two (.1 g, .5 g, 1.0 g, and none). Each flask was then left with a stopper over night. The next day an air sample from each flask was quickly removed and run through a gas chromatograph. This was repeated for three days for consistency.

Results

Experiment One

The Scenedesmus dimorphus grew the best in the 50% autoclaved wastewater (AWW) media. As seen in Figure 2, the algae in the 50% AWW clearly grew better than the other media; thus, it was used as the media for all of experiment two. The autoclaved wastewater did better than the basal media because it started with a higher nutrient content. The raw wastewater preformed the worst because it was taken over by a wild strain of algae that didn’t grow as dense as S. dimorphus.
Figure 2: It is clearly shown that the 50% Autoclaved solution preformed the best. The lines are only added to guide the eye.

**Experiment Two**

As shown in Figure 3, adding sodium bicarbonate to algae cultures increases growth. It was found that the pH of each culture also increased to the limit of the testing paper. Figure 3 was made by averaging the concentration of all four of the PBRs for each level of feeding (e.g. the concentration of all tubes that were given 0.1 g/day). It can be noticed that the concentration of the 50% AWW in Figure 2 is greater than the 0.0 g NaHCO₃ in Figure 3. This is because the wastewater was taken at different times from the source and because the initial concentrations differed between experiments one and two. From Figure 4, one can see that the Cylindrical PBR got better results than the tubular PBR. It was originally thought that the opposite would be true because mixing by the bubbles would be better with the smaller diameter tubes. The bubbles, however, were not strong enough to keep the algae from settling to the bottom out of reach of manual mixing. The manual mixing of the cylindrical PBR led to higher concentrations because the algae that sank to the bottom was mixed before the measurement was taken.
Growth of algae with NaHCO₃ added

<table>
<thead>
<tr>
<th>Concentration [gram/liter]</th>
<th>Day 1</th>
<th>Day 3</th>
<th>Day 5</th>
<th>Day 7</th>
<th>Day 9</th>
<th>Day 11</th>
<th>Day 13</th>
<th>Day 15</th>
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<tbody>
<tr>
<td>0.0 gram/day</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
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<tr>
<td>0.1 gram/day</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.5 gram/day</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.0 gram/day</td>
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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Figure 3: This shows that adding sodium bicarbonate does help growth. There is a clear trend that more bicarbonate leads to more growth. The lines are added to guide the eye only.

Experiment Three

The results of experiment three showed that adding NaHCO₃ to the flask did increase the amount of CO₂ in the air. By partial pressures assuming equilibrium conditions in the flask, the concentration of dissolved CO₂ had to have increased as well. This confirmed that adding sodium bicarbonate should increase the CO₂ provided to the PBRs in experiment two thus increasing the growth of the algae cultures.
Conclusion

There are many drawbacks to the current fuel supply including that it is running out, and that it puts greenhouse gases into the atmosphere. The problems with oil and fossil fuels have led to interest into bio-fuels, those produced from biomaterial; however the current biomaterials have issues as well. These issues are that corn, the main energy crop, is also used for food, which means competition for land. Another issue is that corn is inefficient. It takes more energy as an input than is retrieved from burning the fuel. Current problems have lead into a look at algae, which alleviates many troubles. Algae are not a food sources and are easier to convert and more efficient as fuel.

The experiments done thus far were focused on growing algae efficiently. From experiment one it was discovered that Scenedesmus Dimorphus grew the best in a 50% autoclaved wastewater media. Experiment two showed that adding NaCO₃ to the PBRs, thus increasing the amount of supplied CO₂ to the algae, improved growth. From this research it can be seen that S. dimorphus will tolerate conditions of growing in wastewater, which is necessary while working with anaerobic digestion. There are still a few research questions to answer though concerning how well S. dimorphus grows depending on pH, or if the sodium ions left over in the solution of experiment two had effects on algae growth. In the case of experiment two, would salt water algae be a better choice over S. dimorphus which is a fresh water strain.

As far as anaerobic digestion is concerned related to the research goals, the next projects will be to design and build an anaerobic digester and connect this to cultures of algae. The challenge will be to find the appropriate time to reload the digester and to harvest the algae. Work needs to
be done to test how the systems work together in practice instead of just on paper. First a small system will be used as a model for a larger or full scale anaerobic/algae system.

Works Cited


Acknowledgements

NSF REU

NSF CMMI-0836610
Appendix

<table>
<thead>
<tr>
<th>Sample ID</th>
<th>Total N ppm</th>
<th>Total P ppm</th>
<th>NH4-N ppm</th>
<th>NO3-N ppm</th>
<th>Ortho P ppb</th>
<th>TSS mg/L</th>
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<td>Rww</td>
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<td>109.47</td>
<td>0.03</td>
<td>12642</td>
<td>NS</td>
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Table 1: This is the results from the KSU soils lab on the initial autoclaved and raw wastewater samples used in experiment 1.

<table>
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<th>Sample ID</th>
<th>Total N ppm</th>
<th>Total P ppm</th>
<th>NH4-N ppm</th>
<th>NO3-N ppm</th>
<th>Ortho P ppb</th>
<th>TSS mg/L</th>
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<td>920</td>
<td>NS</td>
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<td>97.4</td>
<td>24.9</td>
<td>0.16</td>
<td>0.02</td>
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<td>NS</td>
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<td>0.02</td>
<td>1723</td>
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Table 2: This shows the results from the KSU soils lab of a sample of each PBR from experiment 2. The samples were taken halfway through the experiment.

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<th>Total P ppm</th>
<th>NH4-N ppm</th>
<th>NO3-N ppm</th>
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<th>TSS mg/L</th>
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</thead>
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<td>12.10</td>
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<td>0.06</td>
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<td>0.29</td>
<td>0.14</td>
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<td>NS</td>
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Table 3: These are the results from the KSU soils lab of samples taken from each PBR at the conclusion of experiment 2.
Proteomics approach for predicting retention behavior of a mixture of proteins during hydrophobic interaction chromatography

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Abstract

Recombinant protein production has increased significantly in recent years, with downstream purification being the most costly part of the overall process. One reason for this stems from a lack of knowledge of the selected host cell protein's (HCP) separation behavior (retention time) during downstream purification. The selection of the downstream process as well as the host cell can benefit from an accurate prediction of the HCP retention time, thereby reducing the resources needed to investigate both. The predicted retention times were estimated using a method where three of the most common protein properties controlling downstream separation were obtained by aqueous two-phase partitioning (surface hydrophobicity) and 2D electrophoresis (pI and molecular weight). Once the protein has been "mapped-out" by the three properties, a partial least squares model was generated using the statistical software program JMP with each property as an independent variable and retention time as the dependent variable. The accuracy of predicting protein retention times using this method has been verified for ion exchange chromatography using a set of model proteins. This paper focuses on obtaining a model for hydrophobic interaction chromatography (HIC), again using a set of model proteins whose retention times have been reported in the literature for three types of resins: Butyl Sepharose, Butyl 650M, and Phenyl 650M. The results show a good correlation for all three resins when using the PLS model as described, indicating the ATPS-2D method can potentially be applied to predict retention times of complex mixtures of HCP during HIC.

Introduction

Recombinant protein production and purification has seen a significant increase in recent years with applications in a wide variety of scientific fields. The most costly aspect of the entire process deals with the purification or downstream processing of the recombinant protein from the contaminating host cell proteins (HCP). One way to decrease this cost is by understanding the separation behavior of the HCP thereby allowing for a more efficient selection of not only the downstream purification method but also the host cell. A way to quantify separation behavior is by determining the amount of time the protein spends bound to the resin before eluting from the column during chromatography, also known as retention time. Chromatography is an expensive as well as time and sample consuming process, therefore a model that accurately predicts retention times of the individual proteins present in complex mixtures such as HCP would help increase the efficiency of purifying the recombinant protein of interest from the HCP contaminants.

The protein properties most commonly used to separate proteins during downstream purification are molecular weight (MW), charge, and surface hydrophobicity (SH). Therefore, these properties would intuitively need to be present in any model aimed at accurately predicting retention times. The next step would be to select simple and relatively inexpensive experimental methods that allow for the determination of the three properties of individual proteins present in a complex mixture.
The method chosen to characterize a protein’s SH is partitioning in an aqueous two phase system (ATPS), which is a bi-phasic system consisting of water, a polymer and a salt with the latter two at immiscible concentrations. After phase separation, the polymer is concentrated in the top phase along with the more hydrophobic proteins and the salt is concentrated in the bottom phase along with the more hydrophilic proteins. Two advantages of selecting this method are that only two fractions result after separation for characterizing and that it is done under non-denaturing conditions. The partition coefficient (K) is the ratio of the protein concentrations in the two phases and the term selected to quantify the protein’s SH. As shown in equation 1 below, a negative Log K represents a higher concentration in the bottom or salt-rich phase and vice versa if the Log K value is positive.

\[
\text{Log } K = \frac{\text{Protein concentration in the top phase}}{\text{Protein concentration in the bottom phase}}
\]

Equation 1

The remaining two properties (MW and charge) are determined using 2-dimensional electrophoresis (2DE) which is a two stage separation where the first is based on the proteins pI or isoelectric point and the second on MW allowing for the separation of a complex mixture of proteins[1]. The isoelectric point of a protein is the pH at which the protein has no net charge and will be the property used to characterize charge in the model. After running the three dimensional (3D) characterization method described here (combining the two characterization techniques of ATPS & 2DE in series), each individual protein originally present in a complex mixture (HCP) will now have all three separation properties characterized (SH, pI, MW).

The 3D characterization method has previously been performed on various HCP samples such as soybean and corn [2, 3]. However, the data has never been modeled for the purpose of predicting retention times during a downstream separation process. There have been studies focused on modeling retention behavior that use a larger number of properties to characterize the proteins [4, 5]. The disadvantage in using larger numbers of properties is that in order to apply the resulting model to the recombinant protein that is the target of the purification, the protein’s crystal structure must be known which is not always the case. A model for predicting retention times using the three properties described here (SH, pI, MW) has been verified as an accurate alternative when compared to the same type of model using the larger number of properties based on using a set of known model proteins with documented properties for a specific downstream purification process (ion exchange chromatography or IEC)[6]. As a result, the goal here is to verify the same idea for a different purification process, hydrophobic interaction chromatography (HIC), again by testing the model’s accuracy against a set of known model proteins. Since this study is focused on verifying that using just the three properties will lead to a model that can accurately predict retention times, this allows for the use of the literature values for pI and MW of the model proteins to be tested instead of obtaining the values individually using 2DE. The hydrophobicity component (Log K) however must still be determined experimentally using ATPS.

Of the various types of downstream purification processes available, one of the more commonly used and non-denaturing methods is HIC providing the justification for modeling HCP retention behavior. The separation of the protein mixture during HIC is based on the binding between hydrophobic areas on the proteins surface and the non-polar ligands on the resin.
in a high salt environment. This study focuses on modeling retention behavior of a set of model (known) proteins during HIC using three different resins: phenyl 650M, butyl 650M and butyl sepharose, all of which have hydrophobic ligands as functional groups.

Materials & Methods

Sodium phosphate monobasic monohydrate, sodium phosphate dibasic heptahydrate, sodium chloride, sodium sulfate anhydrous were purchased from Fisher Scientific (Fair Lawn, NJ). Polyethylene glycol (PEG, MW=3350) was purchased from Sigma-Aldrich (St. Louis, MO). Table 1 below shows the proteins used, all purchased from Sigma-Aldrich, with corresponding MW and pl from noted literature sources.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Source</th>
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</thead>
<tbody>
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<td>8.97</td>
</tr>
<tr>
<td>a-Chymotrypsin</td>
<td>Bovine Pancreas</td>
<td>25</td>
<td>8.75</td>
</tr>
<tr>
<td>Ribonuclease A</td>
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<tr>
<td>Ribonuclease B</td>
<td>Bovine Pancreas</td>
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<td>9.6</td>
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<td>Pyruvate Kinase</td>
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<td>44.287</td>
<td>4.9</td>
</tr>
<tr>
<td>Lipase</td>
<td>Mucor Meihei</td>
<td>29.554</td>
<td>4.81</td>
</tr>
<tr>
<td>β-Lactoglobulin A</td>
<td>Bovine Milk</td>
<td>18.363</td>
<td>4.93</td>
</tr>
<tr>
<td>α-Lactalbumin</td>
<td>Bovine Milk</td>
<td>14.175</td>
<td>4.5</td>
</tr>
<tr>
<td>Lysozyme</td>
<td>Chicken Egg White</td>
<td>14.4</td>
<td>11.35</td>
</tr>
</tbody>
</table>

Table 1: Protein specifications. All proteins were individually prepared in stock solutions at pH 7.0 in 20mM sodium phosphate at a concentration of 5 mg/mL before adding to ATPS system.

- Information from Sigma-Aldrich product information sheet unless otherwise noted
- Information from ExPASy Proteomics Server of Swiss Institute of Bioinformatics
- Plummer and Hirs (1963)[7]
- Howard (1971)[8]

ATPS system composition was 15.7 wt% PEG 3350, 8.9 wt% sodium sulfate and 3.0 wt% sodium chloride with 20 mM sodium phosphate pH 7.0 added until achieving a final system weight of 4.0 grams[9]. Phase ratio (volume of top phase/volume of bottom phase) remained consistent at 0.86 for all ATPS systems. Protein loading was 0.35mg/g ATPS system with α-chymotrypsinogen A (0.20mg/g ATPS) and lectin (0.04mg/g ATPS) the two exceptions in order to prevent oversaturation of the phases. The final systems were centrifuged using a Sorvall RC6
Plus (Thermo Scientific, Rockford, IL) in 15 mL centrifuge tubes (Corning, Corning, NY) with the top phase captured by pipette and bottom phase by piercing the tube with a 10mL Luer-Lok syringe and 18 gauge 1.5 in needle (Becton Dickinson, Franklin Lakes, NJ) taking care not to disturb the interface between the phases. All protein concentrations were determined using the Pierce BCA Protein Assay Kit microplate procedure (Thermo Scientific).

The modeling or statistical analysis portion was done using JMP 8.0 (SAS, Cary, NC) by generating a partial least squares (PLS) linear regression equation of the two forms shown below in equations 2 and 3,

\[
(DRT) = a_1 \log(K) + a_2 \log(MW) + a_3 (\pi - pH)
\]  
Equation 2

\[
(DRT) = a_1 \log(K) + a_2 \log(MW) + a_3 \text{Abs}(\pi - pH)
\]  
Equation 3

with pH = 7.0, DRT = dimensionless retention time (after being corrected for gradient delay and column dead time), Log K = proteins SH component, Log MW = proteins MW component and \(\pi-pH\) or Abs(\(\pi-pH\)) = proteins charge component. DRT is then calculated by adding up the three terms described after each one has been multiplied by its respective coefficient (\(a_1\), \(a_2\) or \(a_3\)).

Results

The SH measurement (Log K) along with the other two characterization properties that are independent variables in equations 2 & 3 (Log MW and \(\pi-pH\) or Abs(\(\pi-pH\))) are shown in Table 2. Also shown are the known literature DRT values for the three resins taken from Ladiwala et al. [10].
### Table 2: Protein property statistics and dimensionless retention times which will be fit into a PLS regression model using JMP.

1 Log K values determined experimentally using ATPS procedure

2 Protein stock solutions have a pH = 7.0 (20mM sodium phosphate)

3 Dimensionless retention times (DRT) taken from Ladiwala et al.[10]

Tables 3 and 4 show the resulting coefficients from a PLS regression model for each of the resins. A positive coefficient indicates that property increases a protein’s retention time for that particular resin and opposite for a negative coefficient. The last column in both tables shows the variable importance plot (VIP) which is an indication of the importance of the variables contribution to the model. If the VIP is small (< 0.8) and the coefficient is also small then that is criteria to delete the variable from the model completely as it is not significant[11].

<table>
<thead>
<tr>
<th>Protein</th>
<th>Protein characterization properties</th>
<th>Dimensionless Retention Times (DRT)³</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Log K¹</td>
<td>Log MW</td>
</tr>
<tr>
<td>α-Chymotrypsinogen A</td>
<td>0.481</td>
<td>1.408</td>
</tr>
<tr>
<td>α-Chymotrypsin</td>
<td>-0.435</td>
<td>1.398</td>
</tr>
<tr>
<td>Ribonuclease A</td>
<td>-1.158</td>
<td>1.137</td>
</tr>
<tr>
<td>Ribonuclease B</td>
<td>-1.786</td>
<td>1.167</td>
</tr>
<tr>
<td>Pyruvate Kinase</td>
<td>-2.060</td>
<td>2.375</td>
</tr>
<tr>
<td>Carbonic Anhydrase</td>
<td>-1.747</td>
<td>1.459</td>
</tr>
<tr>
<td>Lectin</td>
<td>-0.902</td>
<td>1.690</td>
</tr>
<tr>
<td>Ovalbumin</td>
<td>-1.740</td>
<td>1.646</td>
</tr>
<tr>
<td>Lipase</td>
<td>0.153</td>
<td>1.471</td>
</tr>
<tr>
<td>β-Lactoglobulin A</td>
<td>-1.702</td>
<td>1.264</td>
</tr>
<tr>
<td>α-Lactalbumin</td>
<td>-1.761</td>
<td>1.152</td>
</tr>
<tr>
<td>Lysozyme</td>
<td>1.970</td>
<td>1.158</td>
</tr>
</tbody>
</table>

\[
\text{DRT} = a_1 \log \text{MW} + a_2 \log K + a_3 (pI-pH)
\]

Table 3: DRT = a₁Log MW + a₂Log K + a₃(pI-pH)

Table 4: Coefficients from the PLS Regression Model for Each of the Resins.

<table>
<thead>
<tr>
<th>Property (Coefficient)</th>
<th>Phenyl 650M</th>
<th>Butyl 650M</th>
<th>Butyl Sepharose</th>
<th>VIP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Log MW (a₁)</td>
<td>0.370</td>
<td>0.541</td>
<td>0.476</td>
<td>1.436</td>
</tr>
<tr>
<td>pI-pH (a₂)</td>
<td>-0.077</td>
<td>-0.108</td>
<td>-0.132</td>
<td>0.782</td>
</tr>
<tr>
<td>Log K (a₃)</td>
<td>0.152</td>
<td>0.175</td>
<td>0.163</td>
<td>1.083</td>
</tr>
</tbody>
</table>
The two models were shown to allow comparison between how well the model fit the actual DRT data using a different form of the pi component (pi-pH vs. Abs(pi-pH)). After analyzing the coefficients and VIP values, Table 3 statistics follow what is expected when using resins with hydrophobic ligands; SH (Log K) and MW (Log MW) both are significant factors in determining retention time whereas charge (pi-pH) is not as significant and right at the limit for deletion from the model. Table 4 on the other hand, using Abs(pi-pH) to characterize a protein’s charge, does not show reasonable results since Log K and Log MW both do not make significant contributions to the model and therefore should be deleted (according to the VIP criteria). Another illustration of this point, the Log K coefficients are negative suggesting that a protein with greater SH would have a shorter retention time for all three resins. By using this line of reasoning, Equation 2 was the best model to compare accuracy with the published DRT values after analyzing numerous other combinations of the three characterization variables (for instance using (Log K)^2 instead of Log K, having two variables for hydrophobicity in the model, etc.).

The predicted values resulting from the PLS regression model were graphed against the literature values published by Ladiwala et al.[10] and shown in Figure 1. All data points falling on the 45° line would show a perfect correlation between the predicted and literature DRT values.

<table>
<thead>
<tr>
<th>Property (Coefficient)</th>
<th>Phenyl 650M</th>
<th>Butyl 650M</th>
<th>Butyl Sepharose</th>
<th>VIP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Log MW (a₁)</td>
<td>0.042</td>
<td>0.071</td>
<td>0.058</td>
<td>0.729</td>
</tr>
<tr>
<td>Abs(pi-pH) (a₂)</td>
<td>0.082</td>
<td>0.139</td>
<td>0.115</td>
<td>1.442</td>
</tr>
<tr>
<td>Log K (a₃)</td>
<td>-0.036</td>
<td>-0.061</td>
<td>-0.050</td>
<td>0.626</td>
</tr>
</tbody>
</table>

Table 4: DRT = a₁Log MW + a₂Log K + a₃Abs(pi-pH)
Figure 1: JMP output of PLS linear regression model comparing fit of predicted DRT to literature DRT for the three resins: phenyl 650M (top), butyl 650M (middle) and butyl sepharose (bottom).

Of the three resins, butyl sepharose shows the strongest correlation between the predicted and literature DRT values followed by phenyl 650M. The overall fit of the data from all three resins suggest that the PLS model developed (equation 2) demonstrates a good correlation between the predicted and the literature DRT values.
Conclusions & Future Work

The results presented demonstrate that the PLS model developed using only the three characterization properties (MW, SH, pi) accurately predicts retention times of proteins when using HIC as the downstream purification method. Therefore, there is reason to suggest that the three dimensional characterization method described (ATPS and 2DE) can be applied to complex mixtures of unknown proteins (such as HCP) during HIC in order to develop a model that can predict the retention time of a recombinant protein expressed in the selected host.

The next stage would be to actually perform the HIC separation on a HCP mixture (corn, soybean, etc.) and perform the three dimensional characterization on the elution fractions in order to develop a PLS model. Once all the host proteins in the elution fractions have been characterized, the model would need to be tested by running a HIC separation on the host and recombinant protein mixture then testing the model for accuracy.

There are alternative characterization methods available, such as using a series of liquid chromatography separations to obtain the three properties: HIC (to obtain SH) followed by IEC (to obtain pi) followed by mass spectrometry (to obtain MW). This method has clear disadvantages to the average proteomics user (cost, time consuming, etc.). Nonetheless, a comparison of the results from the two characterization methods would prove valuable.

References

Scaffold Architecture’s Effect on Preosteoblastic Cultures Exposed to Continuous Fluid Shear

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*School of Chemical, Biological and Materials Engineering and Bioengineering Center
University of Oklahoma, Norman, Oklahoma 73019

Abstract

Flow induced shear has shown to induce adult mesenchymal stem cells to differentiate along the osteoblastic lineage. This study aimed to discover the effect of different internal architectures on cells seeded and cultured using flow perfusion bioreactors with static cultures serving as controls. In order to achieve this, rat mesenchymal stem cells were seeded and cultured on polymer scaffolds made by solvent casting/particulate leaching and spunbonding. These manufacturing methods produced porous foams and nonwoven fiber meshes, two greatly varied scaffold architectures. In order to maintain the architectures as the controlling attribute, the scaffolds were both composed of poly(L-lactic acid), were ~85% porous, and similar surface area to solid volume ratios. In order to investigate the flow field and shear stresses within the scaffolds, flow simulations based on the lattice Boltzmann method were performed. To obtain the 3D internal structure of the scaffolds to create the computational domain in the simulations, high resolution micro-computed tomography was employed. It was found although scaffold architecture does not appear to create significant differences in average shear or distribution of shear stresses, proliferation and differentiation is affected. Flow perfusion cultures demonstrated four to six times higher cellularities and four times higher alkaline phosphatase activity after 8 days of culture. At 4 days of culture however dynamically cultured nonwoven fibers had three times higher scaffold cellularity over dynamically cultured porous foams. Also, alkaline phosphatase activity of dynamically cultured nonwoven fibers were four times higher than that of dynamically cultured porous foams at 4 days. While these differences in cellularity and alkaline phosphatase activity diminished in dynamically cultured scaffolds after 8 days of culture, nonwoven fiber scaffolds cultured dynamically had measurable amounts of calcium denoting a mineralizing extracellular matrix distinctive of a maturing osteoblastic culture.

I. INTRODUCTION

Treatments of trauma or disease damaged bone requiring supplement material currently involve the use of allografts or autografts. Problems including availability of material, donor site morbidity, and infection plague these choices.[1-2] Bone tissue engineering pursues new supplemental alternatives to naturally heal and remodel nonunion fractures in bone tissue. One promising alternative is the combination of mesenchymal stem cells (MSC) and porous polymer scaffolds.[3-10] Through bioreactor design, forces similar to those found in living bone tissue have been exerted on cell seeded constructs in vitro. These designs include spinner flasks, rotating wall vessels, and perfusion systems.[11-12] Perfusion bioreactors mechanically stimulate cells seeded on porous scaffolds by passing media through the scaffolds exposing cells to fluid shear stresses.[9, 13-20] Along with increased nutrient transfer shear stress has been shown to promote cellular proliferation, differentiation and extracellular matrix (ECM) production.[14, 20-21] Extent or type of cellular promotion has been seen to be dependent on the magnitude of the fluid shear stress exerted upon a construct.[18, 20]
Various production methods are used to make porous polymer scaffolds for bone tissue engineering. Some popular techniques of scaffold production are solvent casting/particulate leaching, rapid prototyping, gas foaming, and electrospinning. These methods produce scaffolds of high porosity (~70-90%) that allow for proper oxygen and nutrient transport, but contain diverse internal architectures. These diverse architectures could produce unique internal shear stress environments for flow rates of equal magnitude. Although the use of porous polymer scaffolds has shown to facilitate the proliferation and differentiation of cells and generalizations of internal fluid shear environments of idealized structures have been analyzed, shear stress distributions within scaffold architectures have not been fully investigated and are not well understood.

Estimations of fluid shear stresses in porous scaffolds, until recently, have been created using simplified assumptions about pore structure and velocity profiles of fluid movement. Analytical methods such as these do not take into account the non-ideal micro-structure of actual internal scaffold networks. This only allows for estimates of flow conditions without giving distributions of the shear stresses inside of the scaffold architecture. To surmount these shortcomings Porter et al. imaged trabecular bone using micro-computed tomography (\(\mu\)CT) and calculated local shear stresses with the lattice Boltzmann method (LBM). Since then, \(\mu\)CT in conjunction with fluid dynamics simulations have been used to characterize shear stress distributions for different types of scaffolds.

The goal of this study is to elucidate the significance of porous scaffold architecture on the growth and osteoblastic differentiation of MSCs cultured under flow perfusion. To achieve this we: 1) characterize the fluid dynamic environments of two different scaffold architectures composed of the same material with comparable surface area to solid volume ratios and porosities and 2) identify the effect of scaffold architecture and shear forces on the proliferation and differentiation along the osteoblastic lineage of MSCs seeded on both types of scaffolds and cultured under static or dynamic conditions in a perfusion bioreactor. These goals were approached through combined computational and experimental analysis.

II. METHODS AND MATERIALS

II.a Porous Foam Scaffold Manufacturing: Porous foam scaffolds were prepared by the solvent casting/particulate leaching method. Briefly, poly(L-lactic acid) (PLLA, 114,500 MW, 1.87 PDI, Birmingham Polymers) was dissolved into chloroform 5% w/v then poured over a bed of sodium chloride crystals (NaCl, 250-350 \(\mu\)m). Solvent was evaporated for 24h resulting in a salt-polymer composite. The composite was inserted into an 8 mm diameter cylindrical mold. The composite was then compressed at 500 psi and heated to 130 °C. Temperature and Pressure was held constant for 30 min. The resulting composite rod was cut into 2.3 mm thick discs using a diamond wheel saw (Model 650, South Bay Technology, Inc). NaCl was leached out of cut discs with deionized water \(\text{(DIH}_2\text{O})\) under agitation for 2 days. Leached discs were then placed under vacuum to remove moisture from the scaffolds. The resulting product was ~85% porous, 8 mm diameter, 2.3 mm thick discs.

II.b Nonwoven Fiber Mesh Scaffold Manufacturing: Nonwoven fiber mesh scaffolds were constructed using PLLA micro-fibers produced by spunbonding. During spunbonding, a hot polymer melt is extruded from a heated die and then fed through a high speed air venturi to attenuate the polymer strand to a fine diameter fiber. The polymer used in the production of fibers was PLLA (grade 6251D, 1.4% D enantiomer, 108,500 MW, 1.87 PDI, NatureWorks LLC.) In the present work, a custom Brabender extruder was used to melt and pressurize the polymer. The polymer exiting from the
extruder was then fed to a modified Zenith pump which pumped controlled quantities of molten polymer through a heated die which has a single polymer capillary of 0.420 mm inside diameter. Polymer flow rates were varied from 0.13 to 0.81 g/min. The polymer strand exited the die and was fed through an air venturi 100 cm below the die nozzle. Resulting fibers were collected on a manually circulated screen placed 175 cm below the die face. This procedure resulted in random nonwoven fibers. Layers of fibers were stacked, measured, and center cut according to de Rovere and Shambaugh’s procedure.[37] Discs were then punched from the layered fiber sheets using an 8 mm diameter die. The resultant scaffolds were ~85% porous with an 8 mm diameter and ~2.3 mm thickness. Collected fibers’ diameters were measured optically using a Nikon HFX-II microscope.

II.c Cell Culture: Adult MSCs were extracted from 175-200 g Wistar rats (Harlan Laboratories).[5, 13, 21, 42] Briefly, tibias and femurs were extracted and the bone marrow was flushed and suspended in α-minimum essential media (α-MEM, Invitrogen) supplemented with 10% fetal bovine serum (FBS, Atlanta Biologicals) and 1% antibiotic-antimycotic (Invitrogen). Cell suspensions were plated on polystyrene 75 cm² culture flasks (BD) and supplemented with osteogenic media (supplemented α-MEM with 50 g/mL ascorbic acid, 10 mM β-glycerophosphate and 10⁻⁶ M dexamethasone (Sigma)). Cultures were incubated at 37 °C with 5% CO₂. Non-adherent cells were discarded after 3 days with osteogenic media being replaced every 2 days. Cultures at ~80% confluency were rinsed with phosphate buffered saline (PBS, Sigma), lifted with trypsin (Invitrogen), centrifuged at 400 g for 5 min, re-suspended in osteogenic media and used for seeding.

II.d Dynamic Seeding and Culture: Cassettes containing pre-wet scaffolds were placed into a flow perfusion bioreactor which has been previously used for culturing MSCs in 3-dimensional scaffolds.[14, 18-20] 200 mL of osteogenic media was then perfused through the scaffolds and bioreactor for 1 hour to rinse and cure the system. Using oscillatory flow perfusion 500,000 MSCs per 0.25 mL of osteogenic media were seeded on each scaffold.[35, 43] After seeding, cassettes containing scaffolds for static culture were removed from the bioreactor and placed in a 6 well plate and submerged in 10.5 mL of osteogenic media. Cassettes for dynamic culture were left in the bioreactor and a continuous unidirectional flow of 0.5 mL/min/scaffold was applied. All cultures were incubated at 37 °C and 5% CO₂ with media being replaced with fresh osteogenic media every 3 days. Seeded scaffolds were cultured periods of 4 and 8 days.

II.e Scaffold Cellularity Analysis: After culturing, scaffolds were removed, washed with PBS and placed in sample vessels with 3 mL of DIH₂O. Foam scaffolds in DIH₂O were crushed into small pieces and fiber scaffolds in DIH₂O were pulled apart. Then samples had 3 freeze/thaw cycles applied to lyse the cells. Using know concentration solutions of λDNA for a standard curve a fluorometric PicoGreen® dsDNA quantification assay (Invitrogen) was used to determine scaffold cellularity. Sample and standard aliquots of 43 μL were placed in individual wells of a 96-well plate along with 20 Å x 10⁻³ M Tris-HCl, 1 Å x 10⁻³ M EDTA, pH 7.5) and 150 μL of Picogreen® dye. Solutions were allowed to incubate in the dark at room temperature for at least 5 min after which using a Synergy™ HT Multi-Mode Microplate Reader fluorescence was measured (490 nm excitation and 520 nm emission). Scaffold cellularity was calculated using the total amount of DNA detected divided by the amount of DNA contained in one cell.

II.f Alkaline Phosphatase Analysis: Alkaline phosphatase (ALP) activity was measured using a colorimetric endpoint assay using p-nitrophenol (Sigma). Increased expression of alkaline phosphatase by preosteoblastic cells indicates cell commitment towards osteoblastic differentiation.[44-47] Scaffolds in DIH₂O used as samples to measure ALP activity. A standard curve was generated from known concentrations of p-nitrophenol. In each well of a 96-well plate, 80 μl of
sample or standard, 20 μl of buffer solution (1.5 M 2-amino-2methyl-1-propanol, pH 10.3) (Sigma), and 100 μl of substrate solution (10 mM 4-nitrophenyl phosphate disodium salt hexahydrate)(Sigma) were added. The plate was incubated for 30 min at room temperature in the dark. The reaction was stopped by adding 100 μl of stop solution (0.1 N NaOH) to each well. A Synergy™ HT Multi-Mode Microplate Reader was then used to evaluate the absorbance (405 nm) in each well. Activity was calculated on a per cell basis.

II.g Calcium Deposition: Calcium content of the cultured scaffolds was measured using ortho-cresolphthalein compound colorimetric method (Kit 587-M) (Sigma) with known concentrations of CaCl₂ used to generate a standard curve. A measureable presence of calcium, a major component of mineralized ECM, gives indication of a maturing osteoblastic culture.[9, 48] Samples previously used in DNA and ALP activity assays were incubated in 0.5 M acetic acid overnight to dissolve any entrapped calcium. For the assay, 10 μL of samples were mixed with 100 μl of calcium binding reagent (0.024% ortho-cresolphthalein complexone and 0.25% 8-hydroxyquinoline) and 100 μl of calcium buffer (500 mmol/L 2-amino-2-methyl-1,3 propanediol and other nonreactive stabilizers) in triplicate in a 96-well plate. The plate was then incubated at room temperature for 10 min. and then the absorbance was read at 575 nm.

II.h Statistical Analysis: Three samples were obtained (n = 3) for all experiments. Values were reported as the average of all samples per experimental group. Reported error was the standard error of the mean. Data analysis was performed using ANOVA with multiple pair-wise comparisons carried out using the Tukey method at a confidence level of 95%.

II.i Scaffold Imaging via SEM: To view polymer scaffold structure, cell morphology, ECM production and cellularity SEM was performed. After culturing, scaffolds were removed from cassettes and washed twice with PBS. The scaffolds were then fixed with 10% PBS buffered formalin (Sigma) at 4 °C overnight. Next, scaffolds were rinsed twice with PBS and passed through serial dehydration using ethanol-water. Samples were then critical point dried using an autosamdri-814 (Tousimis Research Corporation, Rockville, MD), mounted to metal stubs, and sputter coated with gold-palladium using a Hummer VI triode sputtering system (Anatech LTD., Union City, CA). Noncellular scaffold samples were mounted on metal stubs and sputter coated. Imaging was performed on a Zeiss 960 scanning electron microscope (SEM, Carl Zeiss SMT Inc., Oberkochen, Germany) with a tungsten filament. Digital imaging was made possible by the EDS 2008 program (IXRF Systems, Inc., Houston, Texas).

II.j Scaffold Imaging via μCT: Two scaffolds of each architecture were analyzed via μCT by a ScanCo VivaCT40 (ScanCo Medical, Bassersdorf, Switzerland) to obtain 10 μm resolution, 2D intensity image slices at settings of 45 kV energy and 88 μA intensity. Acquired X-ray images were filtered reducing noise and assembled into 3D reconstructions using custom Matlab® code. Scans were segmented using a global thresholding technique which resulted in the porosity of the scaffolds being within 1% of experimentally measured values. Surface area of scaffolds was calculated using 3D reconstructions of the μCT data.[49] Average fiber diameter for the nonwoven fiber mesh scaffolds was verified by 3 different methods: 1) surface area per solid volume ratio using Equation 1; 2) fitting circles to fiber cross-sections on the μCT images using a Matlab® edge detection; 3) optically using a microscope. Area per solid volume or “specific surface area” for cylinders is given as: \[ S = 4/D \] (1) where D is fiber diameter. Average pore size two types of scaffolds was estimated by using their hydraulic radius, \( R_h \). The hydraulic radius was calculated using Equation 2. \[ R_h = \varepsilon/(1-\varepsilon)S_0 \] (2) where \( \varepsilon \) is porosity and \( S_0 \) is the ratio of surface area to volume. These values were obtained using μCT image reconstructions. Hydraulic diameter, \( D_h \), which is a representation of
average pore diameter, was then calculated using: \( D_h = 4(R_h) \) (3) \( R_h \) and \( D_h \) values are reported in

Table 4. A single cuboid portion was obtained from the center of digital reconstructions from each
scaffold. The exact size of the reconstructions was similar with 5.41 mm x 5.41 mm x 1.53 mm for the porous foam scaffold, and 5.41 mm x 5.41 mm x 1.37 mm for the nonwoven fiber mesh scaffold.

II.k Fluid Flow Simulations: Lattice Boltzmann Method: The lattice Boltzmann method (LBM) is a
numerical technique for simulating fluid flow.[50-52] LBM techniques have been used in a wide
spectrum of applications (turbulence[53], non-Newtonian flows[54-56], and multiphase flows[57])
and have computational advantages (e.g., LBM is inherently parallelizable on high-end parallel
computers[58-59]). More important to the present application, LBM is especially appropriate for
modeling pore-scale flow through porous media due to the simplicity with which it handles
complicated boundaries.

A custom-written, in-house code was developed for this work (see Voronov et al.[49] for
further details.) The 3D, 15-velocity lattice (D3Q15) for LBM[60], in conjunction with the single-
relaxation time approximation of the collision term given by Bhatnagar, Gross and Krook[61], was
used to perform simulations. Periodic no-slip boundary conditions were applied at the wall faces in all
directions using the “bounce-back” technique in order to approximate an infinite domain.[51]
Message passing interface was used to decompose the domain in order to take advantage of the
inherent LBM parallelizability. LBM results were validated for flow cases which analytical solutions
are available: forced flow in a slit, flow in a pipe, and flow through an infinite array of spheres.[49]

Calculation of shear stress was conducted following the scheme suggested by Porter et al.[29]
First the full shear stress tensor is calculated, and then the maximum eigenvalue is evaluated using
Jacobi iteration. The cell culture media was assumed to be a Newtonian fluid and to which shear
stresses within a scaffold was estimated as \( \sigma = \mu \left( 0.5 \left( \nabla \vec{U} + \nabla \vec{U}^T \right) \right) \) (4) where \( \sigma \) is the shear stress
tensor, and \( \vec{U} \) is the local velocity vector. 0.01 g/cm s was used for fluid dynamic viscosity, which is
close to that of α-MEM supplemented with 10% FBS typically used in cell culturing experiments.[62]
Velocity vectors used in calculations were derived from flow rates of 0.5 mL/min (used in this study)
and 1 mL/min, both are similar to commonly employed flow rates used for the culturing MSCs in the
flow perfusion bioreactors.[13, 18, 20, 63-64] The shear stresses reported herein are the largest
eigenvalues of \( \sigma \).

III. RESULTS AND DISCUSSION

Table 4: Scaffold comparison based on geometric characteristics

<table>
<thead>
<tr>
<th></th>
<th>Nonwoven Fiber Mesh</th>
<th>Porous Foam</th>
</tr>
</thead>
<tbody>
<tr>
<td>Void Fraction</td>
<td>0.85</td>
<td>0.85</td>
</tr>
<tr>
<td>Surface Area / Solid Volume [cm$^3$]</td>
<td>1046.47</td>
<td>835.52</td>
</tr>
<tr>
<td>Surface Area / Total Volume [cm$^3$]</td>
<td>157.33</td>
<td>125.08</td>
</tr>
<tr>
<td>Mean Diameter From Specific Area [μm]</td>
<td>38.22</td>
<td>-</td>
</tr>
<tr>
<td>Mean Diameter From Edge Detection [μm]</td>
<td>33.01 ± 5.73</td>
<td>-</td>
</tr>
<tr>
<td>Mean Diameter From Microscope [μm]</td>
<td>34.8 ± 1.85</td>
<td>-</td>
</tr>
<tr>
<td>Hydraulic Radius [μm]</td>
<td>54.15</td>
<td>67.82</td>
</tr>
<tr>
<td>Hydraulic Diameter [μm]</td>
<td>216.60</td>
<td>271.28</td>
</tr>
</tbody>
</table>
III.a Polymer Scaffolds

Porous foams made by solvent casting/particulate leaching and nonwoven fiber meshes have distinctively different architectures. The porous foams can be distinguished by their characteristic thin walls and cuboidal pore space structure. The fiber meshes are distinguished by its series of randomly overlaying cylinders. 3D reconstructions revealed highly interconnected porous networks contained within both scaffolds types (Figure 1). The global threshold accuracy chosen for μCT image segmentation was verified by comparison of the diameter of the fibers obtained from μCT reconstructions and the diameter of the fibers measured with other methods (microscope and SEM). Table 4 shows similarities between the two types of scaffolds with a summary of various geometric characteristics.

Table 5: Surface stress calculation results for nonwoven fiber mesh scaffold made by spunbonding and porous foam scaffold made by solvent casting/particulate leaching obtained from LBM for 0.5 mL/min and 1 mL/min flow rates

<table>
<thead>
<tr>
<th></th>
<th>0.5 mL/min</th>
<th>1 mL/min</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Nonwoven Fiber</td>
<td>Porous Foam</td>
</tr>
<tr>
<td>Mean Surface Stress [g/cm²]</td>
<td>0.12</td>
<td>0.13</td>
</tr>
<tr>
<td>Standard Deviation [g/cm²]</td>
<td>0.09</td>
<td>0.11</td>
</tr>
<tr>
<td>Stadard Deviation as % of Mean</td>
<td>74.84</td>
<td>78.18</td>
</tr>
</tbody>
</table>

III.b Shear Stress Distribution within the Pore Space: In order to compare the scaffold types on geometrically equivalent basis, scaffolds were prepared with roughly equivalent specific surface area and volume fraction parameters (Table 4). Summarized results of the surface stresses calculated using LBM are in Table 5.

The apparent lack of difference in mean surface stress values for the two geometries lead to examining the distribution of surface stress. The distribution of shear stresses examined would be that for an idealized initial stage single cell monolayer along the scaffold surface. From the shape of the probability density function (PDF) for the surface stresses inside a porous foam and a nonwoven fiber mesh scaffold (Figure 2) it can be concluded two different scaffold geometries do not display a significant difference in their distribution of shear.
This lack of difference is seen with increased flow rate with an expected elevated mean shear stress.

III.c Scaffold Cellularity: Dynamically cultured scaffolds were observed to have higher cellularity for 4 and 8 day culture periods compared to statically cultured scaffolds (Figure 3). These results are comparable to previously performed studies.[13, 18, 20, 63] Dynamic culturing seems to favor fibers with 3.5 times higher scaffold cellularity at 4 days than dynamically cultured porous foams. Dynamic porous foams seem to overcome this at 8 days of culture with 1.5 times higher scaffold cellularity than dynamically cultured fiber samples. These observations of dynamic cultures may be explained by cells seeded on nonwoven fiber scaffolds ability to easily grow between neighboring fibers allowing for higher initial intercellular connections leading to increased initial proliferation. Interconnected cellular networks are integral to advancing tissue formation beyond the material surface where cells are originally seeded. ECM fixation and migration was probably required for cells seeded upon porous foam scaffolds before intracellular connections and cellular proliferation could occur more rapidly. All statically cultured scaffolds showed no significance in scaffold cellularity.

III.d Alkaline Phosphatase Activity: Alkaline phosphatase activity increases have shown to be a good indication of a commitment towards osteoblastic differentiation.[9, 48] MSCs dynamically cultured on fiber scaffolds were seen to have significantly higher ALP activity, 3.7 to 4.2 times higher, than fiber scaffolds cultured statically (Figure 4). Cells dynamically cultured on porous foam scaffolds did not show a significant difference in ALP activity to static cultured porous foams till 8 days of culture at which they showed 3.6 times higher ALP activity. Flow perfusion after 8 days of culture notably encouraged osteoblastic differentiation of MSCs compared to respective static controls in concord with previous studies.[13, 15, 18, 20, 63]

After 4 days of dynamic culture, cells cultured on fibers were seen to have 3.8 times greater ALP activity over cells on porous foam. When dynamic culture was carried out to 8 days fiber scaffolds did not have a statistical difference (p>0.05) in ALP activity to that of porous foams. These observations align with those seen in cell proliferation data and denote MSCs need to create an ECM network on both types of scaffolds with porous foams requiring a slightly extended period. Once the
ECM is established, scaffolds under flow perfusion appear to be osteoblastically induced predominately by fluid shear forces (emphasized by day 8 ALP data). As expected, no significant difference in ALP activities was seen for all static cultures presumably because the same material, PLLA, was used for all scaffolds.

**III.e Calcium Deposition:** The presence and/or amount of extracellular calcium can indicate the degree of mineralization of deposited extracellular matrix. Maturing osteoblastic cultures can be denoted by mineralized extracellular matrix.[9, 48] No notable amounts of calcium were found for all samples except for 8 day dynamically cultured nonwoven fibers. $225 \pm 7 \, \mu g \, Ca^{2+}/scaffold$ was found for 8 day dynamically cultured nonwoven fiber scaffolds. This calcium presence coincides with the increased ALP activity for the dynamic fibers at 4 days of culture, signifying rapid maturity of the culture. Under similar culture conditions artificial scaffolds have shown little to no calcium deposition at about 8 days.[13, 18, 64] This shows preference by MSCs to quicker differentiation and mineralization under flow conditions on nonwoven fiber mesh scaffolds.

**III.f SEM Imaging:** Visualization of cellularity, ECM production, and cell morphology of cultured constructs was provided by SEM images. Dynamic cultures exhibited extended even surface coverage of cells and ECM deposition. Dynamically cultured cells appeared to have flat cuboidal shapes signifying the morphology of osteoblastic like cells (Figure 5f). Static cultures displayed not only cells with osteoblastic like morphology but cells with rounded appearances signifying morphologies similar to that of MSCs (Figure 5e). Cells were seen to collect in multiple small isolated communities and cells on statically cultured scaffolds. The lack of rounded cells and increased amount of cells and ECM on dynamically scaffolds illustrate the promotion of proliferation and differentiation from fluid shear. Fiber mesh scaffolds dynamically cultured (Figure 5b) appeared to have the majority of cells settled into communities where fibers were contacting or close. Cellular growth penetrating into the interior of pores for dynamically cultured porous foam scaffolds was observed from SEM images (Figure 5d). These ingrowths could potentially lead to clogging which would change the fluid flow characteristics for longer culture periods.

**IV. CONCLUSIONS**

Scaffolds made by solvent casting/particulate leaching and spunbonding with similar porosities and surface area to solid volume ratios do not have a significant differences in average wall shear stress or distribution of shear stresses (shape of probability density function from shear stress) calculated by LBM. Therefore, cell behavior differences observed experimentally can be associated to cells' response to the scaffold's architecture and not to the differences in fluid-induced shear stress exhibited on the cells. Also similar to previous studies, flow perfusion encouraged cellular proliferation and differentiation over that of static cultures.

MSCs seeded onto nonwoven fiber mesh scaffolds made by spunbonding were able to grow to neighboring fibers allowing for higher initial cellular proliferation and quicker differentiation while cells seeded upon porous
9. Sikavitsas, V.I., polymer scaffolds used with flow perfusion. The computational technique applied for this study could be used in other scaffold systems independent of their structural and material properties.

VI. REFERENCES


Targeted Enzyme/Prodrug Therapy for the Treatment of Solid Tumors

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Abstract
A new enzyme prodrug therapy for cancer was tested using human endothelial cells and two breast cancer cell lines in vitro. The concept is to use the human annexin V protein to selectively target the enzyme L-methioninase to the tumor vasculature. The major finding was that enzyme prodrug treatment using the L-methioninase-annexin V fusion protein and selenomethionine as the prodrug over 3 days was shown to be lethal to the endothelial cells and the cancer cells, while having little or no effect with the prodrug but with no fusion protein present. Thus, this new approach appears promising.

1. Introduction
Enzyme prodrug therapy for cancer was conceived as a way to avoid the systemic toxicity of chemotherapy. A type of enzyme prodrug therapy known as antibody-directed prodrug therapy (ADEPT) was first proposed in the 1980’s as a means to confine the action of cytotoxic drugs to the tumor. In ADEPT, a tumor-associated antibody is linked to a drug-activating enzyme, and the resulting fusion protein is administered systemically and preferentially accumulates in the tumor [1]. A non-toxic prodrug is administered systemically and is converted in the tumor to a toxic drug by the enzyme. The enzyme should not have a human homolog to avoid prodrug activation in normal tissues. Although this system is appealing in principle, there have been clinical limitations of ADEPT, including poor accessibility of the enzyme/antibody complex to the tumor [2].

To improve on the ADEPT concept for cancer treatment, we have developed a new approach that targets the enzyme prodrug therapy to the tumor vasculature. In this approach, human annexin V protein is fused to the enzyme moiety and used to bind to the anionic phospholipid phosphatidylserine (PS) on the surface of endothelial cells in the tumor vasculature. PS is typically exposed on the surface of the vasculature endothelium within blood vessels of tumors, but not on normal endothelium [3, 4]. Also, it has been shown that annexin V, when injected systemically, localizes in the tumor vasculature and tumor cells [3, 4]. For the enzyme component, we have chosen L-methionine gamma-lyase (accession #AAB03240), also known as L-methioninase, from Pseudomonas putida. This enzyme catalyzes the α,γ-elimination of L-methionine and selenomethionine and is not found in human tissue [5]. Methionine is converted to methanethiol, α-ketobutyrate, and ammonia, while selenomethionine is cleaved into toxic methylselenol, α-ketobutyrate, and ammonia [6]. Methylselenol has been shown to be approximately 200-fold more cytotoxic to various human cancer cells than its prodrug [7] and is known to induce apoptosis in cancer cells [8, 9].

Mechanisms of action of this new system include: 1) methylselenol generated at the surface of the endothelial cells in the tumor leads to destruction of these cells, leading to clotting in the tumor vasculature and a cutoff of the supply of oxygen to the tumor; 2) methylselenol carried to the tumor cells by fluid permeating through the artery wall because of the pressure gradient across the artery wall; 3) destruction of the endothelial cells and release of tumor antigens directly in the bloodstream, which can cause the immune system to mount a systemic attack on any remaining tumor cells anywhere in the body which can be boosted by the administration of immunoadjuvants [10]. Finally,
the L-methioninase will greatly reduce the supply of methionine in the tumor, which will weaken methionine-dependent cancer cells.

Using the methioninase-annexin V fusion protein as an enzyme prodrug system, here we report the characterization of this system by in vitro studies using human endothelial cells and two breast cancer cell lines that includes a determination of the dissociation constant ($K_d$) of binding to the cell surface, the stability of the binding, and an evaluation of the cytotoxicity of the enzyme prodrug system.

2. Materials and Methods

2.1 Materials

Oligonucleotide primers were produced by the Molecular Biology Resource Facility at University of Oklahoma Health Sciences Center. Linear pET-30 Ek/LIC vector, T4 DNA polymerase, HRV 3C protease, and NovaBlue and BL21(DE3) E. coli cells were obtained from Novagen (Madison, WI). BamHI restriction enzyme and T4 DNA ligase were purchased from New England Biolabs (Ipswich, MA). HAAE-1 endothelial cells were from Coriell Cell Repositories (Camden, NJ). MCF-7 and MDA-MB-231 cells and cell culture media were obtained from the ATCC (Manassas, VA). Streptavidin-HRP was purchased from KPL (Gaithersburg, MD). PCR and plasmid purification kits were from Qiagen (Vista, CA). Alamar Blue solution was obtained from BioSource (Camarillo, CA).

2.2 Construction of Recombinant Expression Plasmid

The expression vector pET-30 Ek/LIC/METHANX, encoding the methioninase-annexin V fusion protein (FP) was constructed in the following manner: The DNA sequences encoding for the FP were amplified from pKK223-3/ATF-Meth \[11\] and pET-22b(+)/STFANX (obtained from Dr. Stuart Lind at the University of Colorado) by PCR using the Expand High Fidelity PCR system (Boehringer Mannheim, Indianapolis, IN). The PCR primers used to create the fusion protein gene, connected by a flexible linker, were as follows:

(a) 5' primer for L-methioninase: 5'- GAC/GAC/GAC/AAG/ATG/CTT/GAA/GTC/CTC/TTT/CAG/GGA/CCC/GCG/GAC/TCC/CAT/ACC/AAC/ACC-3' (b) 3' primer for L-methioninase: 5'- GCA/GCA/ATT/GGA/TCC/AGA/ACC/GCT/GCC/TGC/ACA/CGC/CTC/CAA/GCG/CAG/CTC/G-3' (c) 5' primer for annexin V: 5'- CG/ATT/GGC/GGA/TCC/GCA/CAG/GTT/CTC/AGA/GGC-3' (d) 3' primer for annexin V: 5'- GA/GGA/AA/GCC/CGG/TTA/GTC/ATC/TTC/TCC/ACA/GAG/C-3'. The L-methioninase primers incorporated a 5' LIC cloning site (italics), an HRV 3C protease site (bold), and a 3' BamHI site (underlined). The annexin V primers added a 5' BamHI site (underlined) and a 3' LIC cloning site (italics).

The PCR products were purified using the QIAquick PCR purification kit, digested with BamHI restriction enzyme, and purified. The pure, digested genes were ligated using T4 DNA ligase and run on an agarose gel. Using the QIAquick gel purification kit, the proper fragments were cut from the gel and purified. The pure methioninase-annexin V fusion gene was annealed to the pET-30 Ek/LIC linear vector using T4 DNA polymerase to create sticky ends and was transformed into competent NovaBlue cells. After successful transformation, plasmids containing the proper fusion gene insert were extracted from the NovaBlue cells using the QIAprep plasmid purification protocol and transformed into host E. coli BL21(DE3) for expression. The final vector contains an N-terminal His-tag sequence for easy purification, and an engineered HRV 3C protease cleavage site that cleaves the sequence LEVLFQGV at the start of the methioninase-annexin V gene. The sequence of the FP gene
was verified to be correct by DNA sequencing at the Oklahoma Medical Research Foundation (Oklahoma City, OK).

2.3 Expression and Purification of Recombinant Protein
Recombinant methioninase-annexin V FP was produced and purified using the procedure of Zang [11]. This procedure uses immobilized metal affinity chromatography (IMAC) with immobilized Ni\(^{2+}\) to isolate the FP, followed by cleavage with HRV 3C protease. The purified FP is collected in the flow-through of another IMAC. Any uncleaved FP and HRV 3C protease, which also contains a Histag, remained bound to the IMAC column.

2.4 Protein Content and Enzymatic Activity Determination
The Bradford assay from Bio-Rad (Hercules, CA) was used for all protein determinations using bovine serum albumin (BSA) as the standard. Samples were analyzed by denaturing gel electrophoresis using the SDS-PAGE method with Coomassie blue staining [12]. The L-methioninase enzyme activity was measured using L-methionine as a substrate by the spectrophotometric determination of \(\alpha\)-ketobutyrate with 3-methyl-2-benzothiazolone hydrazone hydrochloride [5].

2.5 Cell Culture
Human HAAE-1 aortic endothelial cells were grown in F-12K medium with 2 mM L-glutamine and 1.5 g/L sodium bicarbonate and supplemented with 10% fetal bovine serum (FBS), 0.03 mg/ml endothelial cell growth supplement, and 0.1 mg/ml heparin. MCF-7 human breast cancer cells were maintained as monolayer cultures in Eagle’s minimum essential medium containing Earle’s balanced salt solution, non-essential amino acids, 2 mM L-glutamine, 1 mM sodium pyruvate, 1.5 g/L sodium bicarbonate, and supplemented with 10% FBS and 0.01 mg/ml bovine insulin. MDA-MB-231 human breast cancer cells were grown in Leibovitz’s L-15 medium supplemented with 10% FBS and 2 mM L-glutamine. Penicillin (100 U/ml) and streptomycin (100 µg/ml) were also added to each medium. HAAE-1 and MCF-7 cells were grown at 37°C in a 5% CO\(_2\) atmosphere, while MDA-MB-231 cells were grown without additional CO\(_2\) at 37°C, as recommended by the American Type Culture Collection.

2.6 Fusion Protein Binding Assay
Each cell line was grown to 70-80% confluence in T-75 flasks. Cells were transferred to 24-well culture plates (5x10\(^4\) cells/well) and grown to 80-85% confluence. Cells were fixed to the plate using 0.25% glutaraldehyde in PBS. Excess aldehyde groups were quenched using 50 mM NH\(_4\)Cl in PBS. Varying concentrations of biotinylated FP, using SureLINK Chromophoric Biotin (KPL) in a 60 molar excess during biotinylation, were diluted in binding buffer containing 0.5% BSA and incubated at 37°C for 2 h. After washing with binding buffer (PBS + 2 mM Ca\(^{2+}\)) with 0.5% BSA, streptavidin-HRP was added at 2 µg/ml and incubated at room temperature for 1 h. Following washing with binding buffer, HRP was measured by adding the chromogenic substrate O-phenylenediamine (0.4 mg/ml) and hydrogen peroxide (0.04 %) in 0.05 mM phosphate-citrate buffer (pH 5.0). After 30 min at room temperature in the dark, the solution was transferred to a transparent 96-well plate, and the absorbance was read at 450 nm on a BioTek Synergy HT microtiter plate reader (Winooski, VT). All experiments had a blank that was subjected to the same procedure but with no FP added. To determine non-specific binding, the same procedure was performed with no Ca\(^{2+}\) and 5 mM EDTA in the binding buffer, with the addition of a 1 h BSA (0.5%) pretreatment for the cancer cells prior to adding the FP.
2.7 Binding Stability Assay
To assess how long the FP remains bound to the surface of the endothelial cells, a modified binding assay was used. Cells on 24-well plates were first incubated for 2 h at 37°C in a saturating concentration of biotinylated FP (100 nM) in complete growth medium with 2 mM Ca²⁺. The Alamar Blue assay (see In Vitro Enzyme Prodrug Cytotoxicity to Cells) was done on separate sets of cells at day 0, 1, 2 and 3 to determine viability, followed by fixing with 0.25 % glutaraldehyde in binding buffer. Excess aldehyde groups were quenched by incubation in a 50 mM NH₄Cl in binding buffer. The binding of FP was then quantified using streptavidin-HRP and OPD as above.

2.8 In Vitro Enzyme Prodrug Cytotoxicity to Cells
The experiment was carried out over 3 days, using the same cells for each of the days. Cells were grown and plated in 24-well plates with respective growth media using the same procedure as for the FP binding assay (see above). Each medium was supplemented with 2 mM Ca²⁺ and 0.02 mM pyridoxal phosphate (since annexin V is Ca²⁺ dependent and pyridoxal phosphate is a cofactor for L-methioninase). On day 0, the cells were incubated in medium containing 100 nM FP for 2 h at 37°C. The plates were washed and medium containing SeMet varying from 0 – 1000 µM was added. The Alamar Blue assay was performed on all wells on day 1. The Alamar Blue assay was performed by adding Alamar Blue solution to each well to give 10% Alamar Blue and then incubated for 4 h at 37°C. The solution was transferred to an opaque 96-well plate, and the fluorescence was read at 590 nm using excitation at 530 nm. The blank consisted of wells containing only medium and Alamar Blue solution. After the fluorescence reading, the plates were washed, replaced with fresh medium containing appropriate levels of SeMet, and placed in the incubator. The readings were taken every 24 hours for the duration of the experiment.

2.9 Data Analysis
All assays included wells in triplicate. To test differences in cell viability, a one-way ANOVA employing a Tukey-Kramer multiple comparisons test was performed using GraphPad InStat software (GraphPad; La Jolla, CA) with a significance level of p < 0.001.

3. Results
3.1 Protein Expression and Purification
An SDS-PAGE gel of the FP purification is shown in Fig. 1. Following expression of the fusion protein, the bacteria cells were harvested by centrifugation, resuspended in lysis buffer and sonicated. The resulting soluble protein fraction is shown in lane 1. The His-tagged fusion protein was bound to the Ni²⁺ column during the first chromatography step to allow unwanted proteins to flow through into waste (lane 2), and then it was eluted (lane 3). After cleavage with the protease, the FP eluted during the flow-through (lane 4) of the second chromatography, while other proteins remained in the column. Dialysis of the flow-through, sterile filtration, endotoxin removal, and lyophilization ended the purification process. The yield of purified FP was 30 mg from 1 liter of culture medium. The purity of the FP (lane 4) was estimated to be >97% using UN-SCAN-IT densitometry software analysis (Silk Scientific, Inc). The theoretical molecular weight of the FP monomer is 80 kDa, which is consistent with the SDS-PAGE result for the purified FP (lane 4). The lyophilized FP was determined to have a methioninase specific activity of 1.0 U/mg of protein. The overall recovery yield of L-methioninase activity was found to be 50%. L-methioninase activity remained relatively constant when stored in lyophilized form at -80 °C.
Figure 1. SDS-PAGE gel of methioninase-annexin V purification. An 8% gel with Coomassie blue staining was used to determine approximate molecular mass of 10 µl of purified samples. Lane 1, soluble proteins; lane 2, first chromatography flow-through; lane 3, first chromatography elution; lane 4, second chromatography flow-through; M marker proteins with molecular masses indicated in kiloDaltons (5 µl).

3.2 Specific Binding and Dissociation Constant Determination
The ability of the FP to bind to endothelial cells and breast cancer cells with PS exposed on the cell surface was evaluated by equilibrium binding experiments in which increasing concentrations of biotinylated FP were used. In initial experiments with endothelial cells, hydrogen peroxide was used at a low concentration (1 mM) to induce exposure of PS. In later experiments, the H₂O₂ was omitted with little change in the results; therefore, the data reported here is with no H₂O₂ added. No H₂O₂ was added in the experiments with the breast cancer lines, since it has been reported that cancer cells express PS in vitro [13, 14]. A typical equilibrium binding result is shown in Fig. 2 for endothelial cells. The non-specific binding, obtained in the absence of Ca²⁺, is subtracted from the total binding to obtain the specific binding. The dissociation constant (K_d) for each cell line tested was obtained from the specific binding data using GraphPad Prism 5 software to give the following results: 0.5 ± 0.2 nM for endothelial cells, 6.2 ± 1.6 nM for MCF-7 breast cancer cells, and 4.9 ± 0.9 nM for MDA-MB-231 breast cancer cells. These results indicate that the binding of the FP to these cells is relatively strong. Literature values of annexin V binding alone to endothelial cells have been reported in the range of 2.7—15.5 nM [15, 16].

3.3 Fusion Protein Binding Stability
The FP bound per cell was studied over 3 days for the three cell lines to determine the stability of binding by measuring the absorbance at 450 nm, determined by the binding assay, and dividing by the fluorescence at 590 nm, determined by the Alamar Blue assay (Fig. 3). The data in figure 3 indicate the binding of FP declined over 3 days for all three cell lines, with the MDA-MB-231 cancer cells showing the most rapid decline; however, the FP was still present at day 3 for all three cell lines. Cell viability, as measured by the Alamar Blue assay, was found to be linearly proportional to the number of cells (data not shown).
Figure 2. Determination of FP binding strength to exposed PS on human endothelial cells. FP was biotinylated and streptavidin-HRP was used to quantify the binding. Total binding was obtained using 2 mM Ca\(^{2+}\) in the binding buffer. Non-specific binding was obtained by removing the Ca\(^{2+}\) from the binding buffer and replacing it with 5 mM of EDTA to chelate Ca\(^{2+}\). Specific binding was obtained by subtracting the non-specific binding from the total binding. GraphPad Prism 5 software determined the specific binding to have a \(K_d = 0.5 \pm 0.2\) nM. Data are presented as mean ± SE (n = 3).

Figure 3. Fusion protein binding stability. The Alamar Blue assay for cell viability was performed each day, followed by the binding assay to determine the duration of binding of annexin V to the exposed PS on the surface of each cell line. ABS/RFU is the absorbance at 450 nm, determined by the binding assay, divided by the relative fluorescence units at 590 nm, determined by the Alamar Blue assay. Data are presented as mean ± SE (n = 3).

3.4 In Vitro Cytotoxicity of Enzyme Prodrug
The ability of the enzyme prodrug system to eliminate human endothelial cells and breast cancer cells was evaluated using a saturating concentration of fusion protein, followed by concentrations of SeMet ranging from 0 – 1000 \(\mu\text{M}\) (Figs. 4, 5, and 6). The methionine concentration in the medium was set at a level (1000 \(\mu\text{M}\)) that would not lead to a significant decrease in cell viability because of methionine depletion with FP present. Each of the cell lines metabolized the Alamar Blue to produce a fluorescence that was measured to quantify total cell viability. The fluorescence data from the Alamar Blue assay was expressed as a percentage of the fluorescence for the cells with no FP and 0 \(\mu\text{M}\) SeMet (control). Cells that were treated with different SeMet concentrations but no FP were compared to the control on the same day, whereas cells that had the FP were compared to cells with the same SeMet concentration but no FP on the same day.
Figure 4. Effect of SeMet conversion to methylselenol on HAAE-1 endothelial cells. Cells were grown in medium adjusted to 1000 μM of L-methionine. Cell viability was assessed using the Alamar Blue assay for cell viability and normalized to the control (i.e., no FP and no SeMet). A one-way ANOVA was performed for statistical analysis. Cells treated with different SeMet concentrations but with no FP were compared to the control (no FP and 0 μM SeMet) on the same day, and statistical significance was denoted by # (p < 0.001). Cells treated with the FP were compared to cells with no FP on the same day at the same SeMet concentration, and statistical significance was denoted by * (p < 0.001). Data are presented as mean ± SE (n = 3).

The cytotoxicity results for the endothelial cells are shown in Fig. 4. Treatment with the FP gave significant cell killing for 500 and 1000 μM SeMet at days 1, 2, and 3 (p < 0.001). With no FP present, significant cell cytotoxicity was not observed at the levels of SeMet tested.

Cytotoxicity results for the two breast cancer cell lines are shown in Figs. 5 and 6. For MCF-7 cells with FP present, there was significant killing at days 2 and 3 with 50 - 1000 μM SeMet (Fig. 5, p < 0.001). Cell killing without FP present was not significant on day 3 until the SeMet concentration reached 1000 μM. MDA-MB-231 cells showed a greater sensitivity to the SeMet than MCF-7 and endothelial cells (Fig. 6); significant cell cytotoxicity was observed with the FP present on days 1, 2, and 3 with 10 - 1000 μM SeMet (p < 0.001). Even without the addition of the SeMet, binding of the FP alone produced significant cell killing. With no FP present, cell killing did not occur until the SeMet level was 1000 μM and was relatively small and not statistically significant (p < 0.001).

4. Discussion
In this study, we have shown that a novel FP developed for use in a new enzyme prodrug system binds relatively strongly to the surface of endothelial cells and two breast cancer cell lines. The
$K_d$ values for the two breast cancer cell lines are similar, while the $K_d$ for the endothelial cells is about an order of magnitude lower, indicating much stronger binding. This low $K_d$ value for the endothelial cells is favorable for this enzyme prodrug system that is directed to the tumor vasculature.

Figure 5. Effect of SeMet conversion to methylselenol on MCF-7 breast cancer cells. Cells were grown in medium adjusted to 1000 μM of L-methionine. Cell viability was assessed using the Alamar Blue assay for cell viability and normalized to the control (i.e. no FP and no SeMet). A one-way ANOVA was performed for statistical analysis. Cells treated with different SeMet concentrations but with no FP were compared to the control (no FP and 0 μM SeMet) on the same day, and statistical significance was denoted by # ($p < 0.001$). Cells treated with the FP were compared to cells with no FP on the same day at the same SeMet concentration, and statistical significance was denoted by * ($p < 0.001$). Data are presented as mean ± SE (n = 3).

The binding stability data (Fig. 3) indicate that the FP was still bound to the cells after 3 days. The effectiveness of the enzyme in the FP during the test period is reinforced by the cytotoxicity data for the endothelial cells and two breast cancer cell lines (Figs. 4-6) for the SeMet concentrations at which there was a statistically significant decline in cell viability compared to the same SeMet concentrations with no FP present on the same day (indicated by a *); for this data there was in almost every case a decline in cell viability from day 1 to day 2 and from day 2 to day 3. The medium for the cells was changed at days 1 and day 2, which means that the enzyme would have to produce additional methylselenol for more cells to be killed; if additional methylselenol were not produced, then no methylselenol would be present (since the medium was changed), and additional cell killing would likely not occur.

In the enzyme prodrug test of cytotoxicity for the endothelial cells, which are the primary target in the tumor for this enzyme prodrug therapy, the cytotoxic effect was evident after only one day of treatment at 500 μM SeMet. At either 500 μM or 1000 μM SeMet, there was no effect on endothelial cell viability with no FP present; this indicates that endothelial cells in the normal
vasculature, which will not bind to the FP (since PS is not externalized), will not be affected by these concentrations of SeMet. At SeMet concentrations well below 500 μM, however, the cancer cells will be killed by toxic methylselenol being carried across the artery wall by fluid permeation; the effect of methylselenol on normal cells outside of the tumor is expected to be minimal or none because it will be greatly diluted by the bloodstream before it reaches the normal cells. MDA-MB-231 breast cancer cells were found to be the more sensitive to the enzyme prodrug treatment than MCF-7 breast cancer cells, even producing killing when the FP was bound but no SeMet prodrug was added.

Figure 6. Effect of SeMet conversion to methylselenol on MDA-MB-231 breast cancer cells. Cells were grown in medium adjusted to 1000 μM of L-methionine. Cell viability was assessed using the Alamar Blue assay for cell viability and normalized to the control (i.e. no FP and no SeMet). A one-way ANOVA was performed for statistical analysis. Cells treated with different SeMet concentrations but with no FP were compared to the control (no FP and 0 μM SeMet) on the same day, and statistical significance was denoted by # (p < 0.001). Cells treated with the FP were compared to cells with no FP on the same day at the same SeMet concentration, and statistical significance was denoted by * (p < 0.001). Data are presented as mean ± SE (n = 3).

Several advantages of this new enzyme prodrug are envisioned. First, the FP can be easily administered through i.v. injection, and it will bind rapidly to its PS receptor in contact with the bloodstream. Second, there would be minimal side effects since the drug is produced locally in the tumor and the prodrug concentration would be at a level that would not affect cells in other tissues besides the tumor. Third, it allows rapid targeting of metastatic tumors anywhere in the body. Fourth, it combines enzyme prodrug therapy with methionine-depletion therapy. Finally, the killing of endothelial cells in the tumor vasculature will cause the release of tumor antigen directly into the bloodstream, leading to a response by the immune system against cancer cells throughout the body, which can be boosted by the administration of immunoadjuvants [10]. One disadvantage, common to all enzyme prodrug systems, is that the enzyme is not a human protein and thus may cause an immune response. This can be overcome by conjugating the L-
methioninase part of the FP to polyethylene glycol (PEG). L-methioninase has, in fact, been conjugated to PEG, and the administration of PEG-L-methioninase to monkeys has been shown to eliminate anaphylactic reactions [17]. In conclusion, based on the data presented, there is evidence that this therapy will be able to cause cytotoxicity to tumors wherever they occur in the body with minimal side effects. This new enzyme prodrug therapy to treat cancer appears promising.

5. Acknowledgement
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6. References
Markovian Modeling and Monte Carlo Simulation of Bacterial Disinfection: Non-Linear Approach

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Abstract

The disinfection of bacterial populations in fluid media entails the elimination or attenuation of vast numbers of microorganisms. These microorganisms are discrete and mesoscopic, exhibiting incessant and irregular motion as well as convoluted non-linear behavior, and colliding frequently among themselves or with the surrounding vessel surfaces and/or mixing devices. Hence, it is highly likely that some of the attributes of the bacteria during disinfection, e.g., their number concentration, will exhibit random, or stochastic, fluctuations as time progresses. Such fluctuations are particularly pronounced at the termination stage of disinfection when the number of bacteria is minute. The exploration of the resultant random fluctuations via stochastic paradigms, therefore, might be profoundly insightful; nevertheless, relatively little has been done hitherto in this regard. The current contribution aims at formulating a Markovian stochastic model for the rate of bacterial disinfection based on a highly non-linear intensity of transition. The resulting master equation of the model has been simulated via the Monte Carlo method to circumvent the complexity of solving it analytically or numerically by conventional numerical techniques. For illustration, the mean, variance (standard deviation), and coefficient of variation of the number concentration of bacteria during disinfection have been estimated through Monte Carlo simulation. The results of simulation are in line with the available experimental data as well as with those computed from the corresponding deterministic model.

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Evaluation of Different Agricultural Feedstocks for Bioethanol Production
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Evaluation of different biomass energy crops for ethanol production is imperative for commercial feasibility of the process. The composition of these bioenergy crops vary and thereby have an effect on saccharification and final ethanol concentration. In our study, five different bioenergy crops: wheat straw (*Triticum aestivum*), forage sorghum stover (*sorghum bicolor*), switchgrass (*Panicum virgatum*), miscanthus (*Miscanthus giganteus*) and sweet sorghum bagasse were evaluated for bio-ethanol production at 20% (w/v) initial substrate concentration. The substrates were ground to less than 600 mesh size and treated with 2% (w/v) NaOH at 121 °C for 30 minutes. The washed and neutralized pretreated residues were subjected to saccharification using cellulase and β-glucosidase enzymes (ratio 1:1.25) at concentrations of 25 fpu/g and 31.25 fpu/g, respectively, in pH 5.0 citrate buffer in an orbital incubator shaker at 150 rpm for 48 h. The hydrolysate obtained was centrifuged and supernatant was collected for fermentation. The fermentation was performed in shake flasks using *Saccharomyces cerevisiae* at 10% (w/v) inoculum concentration at 100 rpm for 24 hrs. Alkali treatment was effective in delignification of all the biomass feedstocks; highest percent removal on raw biomass basis was attained for sorghum stover BMR-DP (81.70%, w/w) followed by miscanthus (80.39%, w/w), sorghum stover BMR-RL (69.83%, w/w), wheat straw (67.45%, w/w), switchgrass (65.43%, w/w) and sorghum bagasse (65.07%, w/w). The highest glucose yield was obtained from sorghum bagasse (0.51 g/g treated biomass) and the lowest was obtained with miscanthus (0.32 g/g), which suggests significant difference in glucose yield for different biomass sources considered in our study. A maximum final ethanol concentration of 3.85% (w/v) was observed for sorghum bagasse followed by wheat straw (3.27%), switch grass (3.26%), miscanthus (2.76%), sorghum DP-BMR (2.71%), and sorghum RL-BMR (2.56%). The results clearly show the final ethanol concentration varies with biomass source, and the quality and quantity of lignin present in the biomass plays a significant role in the saccharification and fermentation steps.
Classification of ketoacyl synthases by their primary and tertiary structures

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Ketoacyl synthases (KS's), encompassing 16 acyltransferases in the EC 2.3.1 classification, are enzymes that condense malonyl-ACP or malonyl-CoA with acyl-ACP or acyl-CoA units. This condensation reaction forms new carbon-carbon bonds, and it is a key step in the fatty acid elongation cycle. Here, our classification of KS's according to their primary and tertiary structures instead of by substrate and reaction catalyzed provides some insights into this enzyme group in terms of its members' tertiary structures, catalytic residues, and mechanisms. A series of computational methods including BLAST, multiple sequence alignment, tertiary structure superposition, and root mean square calculations are used to classify them into different families and clans. At present, there are 21 potential families according to primary structure analysis by BLAST and multiple sequence alignments. The tertiary structures of KS's and their phylogenetic trees will be further analyzed in the near future.
Catalytic domain organization in hydroxyacyl dehydratases, enoyl reductases, and ketoacyl reductases

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Hydroxyacyl dehydratases (HDs) are classified from EC 4.2.1.1 to EC 4.2.1.120 based on their catalytic activities on different substrates. Those active on substrates containing thioester groups can be classified into six families whose members have related amino acid sequences (primary structures). On the other hand, members of different families have mainly unrelated primary structures, although those in families 1 and 3 have similar three-dimensional (tertiary) structures and catalytic mechanisms, and therefore they can be grouped into a superfamily or clan. The six families contain enoyl-CoA hydratases, fatty acid synthases, and hydroxydecanoyl-[acyl-carrier-protein] dehydratases. For enoyl reductases (ERs), preliminary results suggest that they can be grouped into nine families containing flavin oxidoreductases, short chain dehydrogenases, 2-nitropropane dioxygenases, alcohol dehydrogenase GroES-like domains, zinc-binding dehydrogenases, and 3-oxo-5α-steroid 4-dehydrogenases. For ketoacyl reductases (KRs), the preliminary results show they can be grouped into seven families containing alcohol dehydrogenases, 3-hydroxyacyl-CoA dehydrogenases, hydroxymethylglutaryl-CoA reductases, and 3-oxoacyl-[acyl-carrier-protein] reductases. The primary and tertiary structures of HDs, ERs, and KRs are included in the new ThYme (Thioester-active enzYmes) database.
Molecular Dynamics Investigation of the Effects of Ionic Strength on LCST of Poly (N-isopropylacrylamide)

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Abstract

Thermal responsive membranes can be used as anti-fouling membranes for water treatment and for self-cleaning purposes. Poly(N-isopropylacrylamide) (PNIPAAm) is a thermo-responsive polymer which has a corresponding low-critical solution temperature (LCST) of 32°C in water. Ionic strength, pH and the chain length are found to have a significant effect on its LCST value. Molecular dynamics (MD) simulations were carried out for a 50 monomer unit PNIPAAm to investigate the effects of ionic strength on its LCST using NAMD simulation package with Amber force field. The effect of ionic strength on LCST was studied by investigating the polymer conformational change in NaCl, NaBr, NaI, KCl, KBr and MgCl₂ aqueous solutions with varying ionic strength at different temperatures around their corresponding LCST values. Good agreement with experimental LCST data was achieved. Moreover, the contributions to the stability of the polymer structures above and below LCST due to electrostatic, hydrogen bonding and van der Waals interactions were analyzed.
DEVELOPMENT OF A BIOREACTOR SYSTEM FOR THE CULTIVATION OF AN ENGINEERED PERIODONTAL GRAFT

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Perfusion culture of oral mucosa substitutes presents a unique challenge due to a wide range of loading conditions on these tissues. In this study, the potential of a unique 3D ex-vivo scaffold derived from the human umbilical vein (HUV) as an oral grafting material cultured in a novel pulsatile bioreactor system that mimics the oral environment is investigated. Briefly, HUVs are isolated from umbilical cords using a semi-automated machining technology and decellularized through an optimized protocol incorporating 1% (wt/vol) sodium dodecyl sulphate (SDS). The scaffolds are reseeded with human gingival fibroblasts (hGFS) and cultivated under dynamic conditions. Dynamic compressive properties of the cultured HUV grafts are examined to gain insight into the effect of mechanical stimulation on in vitro engineered constructs. Biological responses (cell attachment, viability and proliferation, and differentiation of the phenotype of the HUV) at 0, 7, and 28 days are also assessed.

Poster presentation
Regulation of focal adhesion maturation and cell edge dynamics by epidermal growth factor

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Cell migration plays an essential role in many biological processes, such as cancer metastasis, wound healing, and immune response. Cell migration is controlled by a series of feedback loops. One such feedback loop exists between adhesion and protrusion. Adhesion is mediated through focal adhesions (FAs), which are dynamic, macromolecular structures that serve as mechanical linkages and centers of intracellular signal transduction. FAs exist in different maturation states. These maturation states are defined by FA size and protein density, and they regulate whether protrusion or retraction is promoted. Epidermal growth factor (EGF) can regulate protrusion as well; however, it is not known whether this is through FA maturation. We are using total internal reflection fluorescence microscopy techniques to examine the spatial and temporal correlation between FAs and protrusion in a rat mammary adenocarcinoma cell line under different doses of EGF. We have found that cell speed and edge dynamics have a biphasic relationship with EGF dose. Additionally, the number of FAs was monotonically dependent on EGF concentration, whereas the size and protein density was inversely correlated with cell speed and edge dynamics. These results strengthen the hypothesis that EGF regulates cell migration through FA maturation.
Bio-chemical functionalization of graphene for cancer cell detection

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Electronic interfaces fabricated using nanomaterials offer exciting avenues for the development of improved biosensing devices. The 2-D nanomaterial Graphene, has received tremendous research attention in recent years that is attributed to its sp²-hybridized surface construct, which is atomic scale thick and yet is multi-micron scale large. While its atomic thickness imparts electronic confinement of the high-density charge carriers, the large modifiable surface area provides excellent avenues for detecting molecules via surface binding. However, the available surface modification schemes for bio functionalization involve covalent bond formations, which introduce several defects in graphene by removing its π-electrons and introducing vacancy defects, significantly degenerating graphene's superior properties. In this talk, we will present a novel approach for interfacing graphene with biological and physical molecules via non-covalent bonds that introduce molecular recognition specificity as well as retain the superior electrical properties of graphene. We will present the functionalization of several biomolecular groups on graphene, characterize their effect on its electrical properties and discuss the conjugate’s detection properties for enhanced biosensing of Circulating Tumor Cells (CTCs). We envision that by leveraging the non-covalent chemistry on graphene, a novel class of defect free graphene-hybrids can be fabricated with tunable physico-chemical and electrical properties for advanced applications in biological/chemical sensing, nanoelectronics and optoelectronics.
Bioinspired synthesis of calcium phosphate nanocomposites templated by block copolymer-peptide conjugates

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Self-assembling thermoreversibly-gelling block copolymers conjugated to hydroxyapatite-nucleating peptides were used as templates for the growth of inorganic calcium phosphate nanostructures. Specifically, pentablock copolymers with poly(acrylic acid), poly(ethylene oxide), and poly(propylene oxide) blocks were synthesized using atom transfer radical polymerization and used as templates for biomineralization. Characterization of these polymers revealed narrow molecular weight distributions. Hydroxyapatite-binding peptides were conjugated to the ends of the polymer chains. Aqueous solutions of these polymers formed physical gels when the temperature was increased to above room temperature, allowing for formation of self-assembled nanocomposites by biomineralization. Calcium and phosphate ions were dissolved in these block-copolymer aqueous solutions at low temperature. Aging at room temperature produced inorganic nanoparticles. The self-assembled nanocomposites were characterized by nuclear magnetic resonance (NMR), transmission electron microscopy (TEM), X-ray diffraction, thermogravimetric analysis (TGA), and small-angle scattering. It was confirmed that the peptides promoted the formation of hydroxyapatite as the inorganic phase. Nanocomposite formation was confirmed by solid-state NMR. Small-angle X-ray scattering and TEM indicated thin, elongated crystallites. TGA showed an inorganic content of 30-45 wt%, which was based on the mass of the dried gel at about 200°C. Hydroxyapatite was identified as the inorganic component by several methods. Our work developed novel, self-assembling, injectable nanocomposite biomaterials, which have promising orthopedic applications. Our bioinspired bottom-up approach offers novel routes for synthesis of other inorganic materials in the future.
Field Investigation of the Bioremediation of Chlorinated Ethenes in Groundwater

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Abstract

The effect of addition of edible oils to the subsurface to promote anaerobic biodegradation has been investigated over a period of 6 months at the Aggieville site located near the southeast edge of the KSU campus. After the addition of edible oils to the aquifer, concentrations of tetrachloroethene (PCE), trichloroethene (TCE), and dichloroethene (DCE) were measured in monitoring wells 7D (MW-7D) and 8D (MW-8D). The contamination source is 100 ft upgradient of MW-7D and MW-8D. MW-8D was part of earlier pilot studies which included injections of soy oil methyl esters (SOME). MW-7D was outside of the treatment zone and was therefore used as a reference. Groundwater flow rate was approximated at ¼ - ½ ft/day. MW-7D was found to be anaerobic 3 months after addition of edible oils 10 feet upgradient. The confirmation of anaerobic conditions in MW-7D showed that the edible oil addition was producing the desired effect of biodegradation of PCE. The measured concentrations of PCE decreased with time because of microbial transformation of PCE to TCE and DCE.
Ultrafast, Label-free detection of Pathogenic Bacteria via Peptide-modified-Graphene bio-sensor

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Rapid detection of pathogenic agents forms the cornerstone of medical diagnostics & treatment and to satiate the present-day concern of bio-defense. In this context, Graphene, the two dimensional elusive nano-material comprising of a highly conducting honey-comb lattice of sp² bonded carbon atoms, shows a lot of promise for bio-sensing owing to the large interfacial area, high sensitivity and exceptional mechanical and chemical stability. Here we leverage the facile modulability of the charge carriers in peptide-modified-Graphene (PMG) with the high biological specificity of peptides / antibodies for an ultrafast, label-free detection of pathogenic bacteria with single bacterium sensitivity. In this proof-of-concept study, Concanavalin-A and a heptapeptide (with a sequence AWLPWAK), which exhibit a strong, specific affinity towards Gram-positive bacteria and E. coli respectively, were covalently attached to the oxy-functionalized graphene via an amide linkage to produce the PMGs. Upon attachment of the complementary pathogenic bacterium, ~ 300 % increase in the PMG channel conductivity was detected which was attributed most-likely to the electrostatic charge mediated gating or to the carrier doping of the underlying graphene. Further, we will also present the electrical studies on non-complementary bacteria in which a modest (< 20 %) change in conductivity vindicates the robustness of the detection system.

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Graphene is a chemically stable, highly conductive single atom thick layer of sp² carbon atoms arranged in a honeycomb lattice, whose atomic impermeability and mesoscale flexibility, makes it the ultimate interfacial material. Here we demonstrate the first ever swaddling of a live bacterium with ~1 nm thick peptide-modified-Graphene: (1) to enable electron microscopy of whole cells with 48 % reduced cellular shrinkage and decreased electron beam damage; (2) to control molecular permeation through the cells, and; (3) to enhance Raman scattering (~300 %) from the cells. Upon interaction with the gram-positive bacterial cells having a complementary surface biochemistry, the peptide-modified-Graphene nano swaddlers (GPNS) instantaneously (< 1 min) wrap the bacterial cells *via* strong and specific interaction with the cell wall moieties. Dye permeation studies confirm the impermeability of the GPNS consistent with the theory. Further characterization of the wrapping process would be presented using concurrent microscopic and spectroscopic analysis. Time-lapse TEM imaging studies on both wrapped and unwrapped bacteria, showed a ~48 % reduction in efflux of the cellular material from the wrapped bacteria, enabling circumvention of the standard lengthy sample preparation protocols. We will also present the Raman spectroscopic results in which a ~300 % chemical enhancement of the scattering signal was observed upon wrapping.
While nanoparticles have a large potential to be used to improve the treatment of cancer, a major barrier of further development is the navigation of the nanoparticle from its point of introduction into the body to its final destination. A new cancer treatment system is being developed with the goal of delivering single-walled carbon nanotubes (SWNTs) to the tumor rapidly and efficiently. This system uses SWNTs that are functionalized with the protein annexin V. This protein binds selectively to anionic phospholipids such as phosphatidlyserine (PS). PS exposure on the outer cell membrane is almost completely exclusive to cancer cells and endothelial cells that line the tumor vasculature. In a treatment of cancer by this system, SWNTs bound to the tumor vasculature would be exposed to near-infrared light (NIR) at a wavelength that heats the SWNTs but not the surrounding normal tissue, resulting in the tumor being destroyed.

In this study, we have covalently coupled the human protein annexin V to a linker that is strongly adsorbed to the SWNTs by a fluorene group in the linker via a $\pi-\pi$ stacking binding. We avoided covalently coupling the protein to the SWNT sidewall to avoid loss of the NIR absorption by the SWNTs. An in vitro system was used for human endothelial cells with PS exposed. The dissociation constant ($K_d$) for the binding of annexin V to the cells was determined. The effect of NIR light on endothelial cells with the SWNT-annexin V complex bound was studied using the Alamar blue assay to evaluate cell viability.

Poster presentation
Development of Fast-acting Microspheres for Thrombolytic Therapy

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Microspheres for fast-acting purpose in thrombolytic therapy are being developed so that their action should be slightly delayed. This microparticulate system uses a combination of acetalated dextran (Ac-Dex) and maleic anhydride (MA). Ac-Dex is prepared by modifying dextran with acetal-protecting groups. Ac-Dex has solubility properties orthogonal to those of the dextran, such that it is only soluble in organic solvents and completely insoluble in water. Under acidic aqueous conditions, the pendant acetal groups of Ac-DEX are hydrolyzed. MA is used as an acid catalyst to hydrolyze Ac-DEX.

In this study, microspheres were characterized by their morphology and particle size. Their protein (BSA) loading efficiency and release profile of BSA in vitro were investigated. Protein release profiles from these microspheres can be controlled by changing the Ac-Dex/MA mass ratio.

Poster presentation
Growth of Algae using Nitrogen and Phosphorus in Wastewater

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Abstract

There are many drawbacks to the current fuel supply such as the depletion of oil, and the pollutants created upon using the fuel. These drawbacks to oil had led to interest into bio-fuels, those produced from biomaterial; however some biomaterials are better than others. The goals of the project are to investigate the effect of wastewater concentration on the growth of Scenedesmus dimorphus (algae), to explore the growth of Scenedesmus dimorphus while being fed differing amounts of sodium bicarbonate daily, and to examine the effect of different photo-bio reactors (PBRs) on the growth of Scenedesmus dimorphus. In experiment one, five different media were used; 50% autoclaved wastewater, rest tap water performed the best. For experiment two, sodium bicarbonate was tested as a fertilizer and was found to be effective in increasing the growth of the algae. Finally, the cylindrical PBRs were found more effective than the tubular PBRs.
Designing Virus Surrogates
Emily Stump, Hailey Cutler, Guadalupe D. Gutierrez, Scott Husson, John Pellegrino, Ranil Wickramasinghe

Viral contamination poses a risk to pharmaceutical and medical products. For example, products such as interferons, monoclonal antibodies, hormones, vaccines, and recombinant proteins are required to be tested and evaluated for viral safety before release to market. Contaminations of biotechnology products with organisms such as bovine viral diarrhea virus, epizootic hemorrhagic disease virus, minute virus of mice, hepatitis B virus, SV40, avian leukemia virus, and vesivirus have been reported previously. In many cases, filtration with membranes (also known as virus clearance filters) is the standard practice to remove virus from fluids. Virus clearance filters must maximize virus rejection, product protein passage, and capacity (i.e., the amount of feed that can be treated per membrane area). It is necessary to verify the performance of the filter in separate viral clearance studies. These studies call for the intentional addition of a known titer of virus (or virus surrogate) to bulk material and subsequent evaluation of the effectiveness of the process to remove that known quantity of contaminant. It is desirable to validate the retention properties of a filter or membrane without utilizing live virus, due to the inherent risks that viruses bring to the environment, personnel, and the manufacturing facility. Additionally, current viral clearance studies are laborious and require highly trained personnel. Viral assay testing is quite time consuming, and there are potential viral contaminants which may not grow in culture or for which we do not have assays. Development of a model, non-biological system to simulate virus particles could be of great practical value as it could significantly reduce the time taken to test new membranes; reduce development costs; and lead to more reliable data, since a non-biological system will reduce variability in the assay. Here we develop a magnetic nanoparticle based system for simulation of 20 nm parvovirus particles, as well as a novel detection system for the virus surrogate.

In this study we have investigated the rejection of minute virus of mice, a model virus, in the presence of BSA, a model protein. Commercially available virus clearance filters and ultrafiltration membranes have been tested. The effect of operating conditions, such as protein concentration, on virus rejection, protein passage, and permeate flux have been investigated. These filtration experiments serve as the basis for comparison to filtration with virus surrogates, 20nm magnetic nanoparticles, and help to identify retention mechanisms that are important to consider for the design of prospective surrogates. Additionally, a prototype of a novel gigantic magnetoresistive (GMR) sensor has been fabricated and tested. This detection method for virus surrogates has the potential to eliminate biological variability in quantitative assays and increase limits of detection.
Proteomics approach for predicting retention behavior of a mixture of proteins during hydrophobic interaction chromatography

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Recombinant protein production has increased significantly in recent years, with downstream purification being the most costly part of the overall process. One reason for this stems from a lack of knowledge of the selected host cell protein’s (HCP) separation behavior (retention time) during downstream purification. The selection of the downstream process as well as the host cell can benefit from an accurate prediction of the HCP retention time, thereby reducing the resources needed to investigate both. The predicted retention times were estimated using a method where three of the most common protein properties controlling downstream separation were obtained by aqueous two-phase partitioning (surface hydrophobicity) and 2D electrophoresis (pI and molecular weight). Once the protein has been “mapped-out” by the three properties, a partial least squares model was generated using the statistical software program JMP with each property as an independent variable and retention time as the dependent variable. The accuracy of predicting protein retention times using this method has been verified for ion exchange chromatography using a set of model proteins. This talk will focus on testing the accuracy of the model for hydrophobic interaction chromatography (HIC), again using a set of model proteins, against retention times taken from the literature for three types of resins: Butyl Sepharose, Butyl 650 M, and Phenyl 650 M. The results show a good correlation among all three resins when using the PLS model as described, indicating the ATPS-2D method can potentially be applied to predict retention times of complex mixtures of HCP during HIC.
Scaffold Architecture’s Effect on Preosteoblastic Cultures Exposed to Continuous Fluid Shear

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Flow induced shear has shown to induce adult mesenchymal stem cells to differentiate along the osteoblastic lineage. This study aimed to discover the effect of different internal architectures on cells seeded and cultured using flow perfusion bioreactors with static cultures serving as controls. In order to achieve this, rat mesenchymal stem cells were seeded and cultured on polymer scaffolds made by solvent casting/particulate leaching and spunbonding. These manufacturing methods produced porous foams and nonwoven fiber meshes, two greatly varied scaffold architectures. In order to maintain the architectures as the controlling attribute, the scaffolds were both composed of poly(L-lactic acid), were \textsim\textasciitilde85\% porous, and similar surface area to solid volume ratios. In order to investigate the flow field and shear stresses within the scaffolds, flow simulations based on the lattice Boltzmann method were performed. To obtain the 3D internal structure of the scaffolds to create the computational domain in the simulations, high resolution micro-computed tomography was employed. It was found although scaffold architecture does not appear to create significant differences in average shear or distribution of shear stresses, proliferation and differentiation are affected. Flow perfusion cultures demonstrated four to six times higher cellularities and four times higher alkaline phosphatase activity after 8 days of culture. At 4 days of culture however dynamically cultured nonwoven fibers had three times higher scaffold cellularity over dynamically cultured porous foams. Also, alkaline phosphatase activity of dynamically culture nonwoven fibers were four times higher than that of dynamically cultured porous foams at 4 days. While these differences in cellularity and alkaline phosphatase activity diminished in dynamically cultured scaffolds after 8 days of culture, nonwoven fiber scaffolds cultured dynamically had measurable amounts of calcium denoting a mineralizing extracellular matrix distinctive of a maturing osteoblastic culture.

Oral presentation
The Use of Targeted Enzyme/Prodrugs for the Treatment of Solid Tumors

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A new anticancer therapy for solid tumors is being developed to eliminate both the primary tumor and distant metastases. This approach uses an enzyme/prodrug system targeted to the solid tumor vasculature. Two different genes encoding bacterial enzymes were linked to human annexin V protein to form fusion proteins (FPs). Annexin V is known to bind with high affinity and specificity to exposed the anionic phospholipid phosphatidylserine (PS) on the outer membrane of endothelial cells in the tumor vasculature. The enzymes are used to activate nontoxic prodrugs into toxic anticancer agents. Following FP administration into the bloodstream, the prodrugs are delivered, and the resulting toxic molecules induce (1) death to tumor endothelial cells leading to vessel clotting and oxygen deprivation, and (2) death to surrounding tumor cells because of leaky capillaries and the enhanced permeability and retention (EPR) effect. Using recombinant bacterial technology, the fusion proteins were expressed and purified from E. coli. Assays were performed to determine the strength of FP binding, cytotoxicity and binding stability for human endothelial cells and breast tumor cell lines MDA-MB-231 and MCF-7. Cytotoxicity assays demonstrating cell killing were done using the Alamar Blue assay. Near complete killing was observed for both the methioninase/selenomethionine and the cytosine deaminase/5-fluorocytosine enzyme prodrug combinations.

Oral presentation
Highly porous uniform pore size membranes for ultrafiltration

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Uniform pore size, highly porous membranes, could find applications in ultrafiltration for fractionation of proteins with molecular weights that differ by less than an order of magnitude, as well as in membrane absorbers as chromatographic support materials. However large scale economical production of these membranes has been problematic. Here we describe the development of three dimensionally ordered macroporous (3DOM) structures, also known as inverse opals. Conceptually, preparation of inverse opal structures is simple: close-packed colloidal crystal templates are infiltrated with chemical precursors that are polymerized, and the template is removed resulting in a 3DOM structure consisting of close-packed spherical voids hundreds of nanometers in diameter separated by polymeric walls tens of nanometers thick. Here, we focus on the development of a new class of ultrafiltration membranes, inverse colloidal crystal (ICC) membranes based on inverse opal structures. ICC membranes could find numerous other applications which require uniform pore sizes.

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Production and characterization of porins

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Mycobacterium Smegmatis Porin A (MspA) will be produced and purified on the bench scale and characterized. For future investigation of MspA and possible applications in nanotechnology as well as research in treating tuberculosis it is necessary to produce, purify, and characterize this unique protein.
Production of Nitrogen-Based Platform Chemical: Cyanophycin Biosynthesis using Recombinant Escherichia coli

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Cyanophycin (muti-L-arginyl-poly-L-aspartate) is a non-ribosomally synthesized reserve polypeptide, which consists of equimolar amounts of arginine and aspartic acid arranged as a polyaspartate backbone and arginine as the side chain. Cyanophycin is an environment friendly chemical that can be converted to a derivative with reduced arginine content, or to a completely biodegradable poly aspartic acid, which can be used as a substitute for non-biodegradable polyacrylates. Biosynthesis of cyanophycin is catalyzed by cyanophycin synthetase. Anabaena Variabilis ATCC 29413 contains the structural gene (cphA) for cyanophycin synthetase, which was cloned and sequenced successfully in this study. The cphA gene was amplified by PCR, ligated to the vector pET45b+ and introduced into E. coli BL21 (DE3) pLysS and E.coli BL21 (DE3). Experiments by design for high cyanophycin synthesis was performed at shake flask level using the recombinant BL21 (DE3)pLysS, LB broth as carbon and nutrient source, and casamino acid as primer. Experiments are ongoing using recombinant BL21 (DE3) pLysS for cyanophycin production at 2 to 5 L fermenter level. Successful outcome of this study will enable a low-cost production method for cyanophycin synthesis, which can be used as a platform chemical to produce other useful derivatives.