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ACKNOWLEDGEMENTS

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We especially express appreciation for Gina Myerson and Kyle Delaney, Director and Assistant Director of Marketing and External Communications at the McCormick School of Engineering and Applied Science, for their eagerness to help make NURJ more of a success. Thanks to Kyle as well for helping us to secure our printer, to Curtiss Haug at Graphic Arts Studio for the print job, and to Patrick Stansbury at Pentagon Publishing.

We would also like to acknowledge members of the Faculty Review Board: Mark Hersam, Allen Taflove, Rick Gaber, Michael Peshkin, Owen Priest, and Ming-Yang Kao. Their expertise is indispensable. To all of the contributors, we are grateful for their desire to share their exemplary research, and to their advisors and mentors, we are just as grateful for inspiration and guidance. With this third issue, special thanks are extended to the contributing authors from the Medill School of Journalism: Joanna Allerhand, Annie Martin, Angela Chang, and Nicole Price Fasig.
To the Northwestern Research Community:

Welcome to the third annual issue of the Northwestern Undergraduate Research Journal. Publication in NURJ is but one avenue for undergraduates to develop their skills as independent researchers. It provides them with a forum to share their work from the laboratory to the broader community. As it is, the Northwestern community is one that encourages its students to learn outside in practice as well as inside the classroom.

I am pleased that NURJ highlights the contributions of faculty to our undergraduate research. One prime example is Dorothea Koh who was inspired in her chemistry class to seek research in the welcoming environment of the Nguyen laboratory. Also, through the NSEC and USS programs, undergraduates work with faculty who dedicate their resources to provide opportunities for experience.

Still, the opportunities are only half of the story. As faculty inspire students, so too does NURJ seek to promote scientific research among undergraduates with examples of their dedicated peers. Highlighted on the cover, Rahul Sawlani's work demonstrates ongoing endeavor in the practical field of cancer research. You will also find in reading about the authors that some of their work is a stepping stone for further ventures.

Finally, with NURJ still in its infancy, I would like to open communication with you, to help us best present the impressive undergraduate research. I hope you will enjoy the articles in this issue.

Sincerely,

[Signature]

Vinhfield Ta
Editor
want to get published?

The Northwestern Undergraduate Research Journal is now accepting submissions for the 2007 Journal.

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Getting Wild

Students Leave the Classroom for Experience in the Field

by Joanna Allerhand

During fall quarter of his junior year, Jake Bledsoe’s alarm was replaced by howler monkeys whose loud and obnoxious calls woke him up at 5:30 each morning. But since he was living in a cabin on the beach, he could step outside for an early view of the Pacific Ocean and start his day off right.

“It was nice just being able to live right there in the environment you’re interested in, especially because they’re so beautiful,” said Bledsoe, a third-year Biology major.

Bledsoe was participating in a study abroad program in Costa Rica for students interested in tropical studies. This program, organized through Duke University, is one of a handful of options available for students interested in science-related field studies.

In Costa Rica, most of the classes were taught in the field, Bledsoe explained. The group of 28 undergraduates, two of them Northwestern students, traveled throughout the country. At each area they visited, professor lectured about the local ecology.

The focus of the program was evolutionary biology and ecology. Participants learned about taxonomy and how to identify the organisms that surrounded them.

“It’s a neat way to get out in nature and see what’s happening first hand as opposed to reading about it,” Bledsoe said.

At two sites, students narrowed their focus and designed their own research projects. Each student spent a week collecting data, which was then analyzed and written up in a report.

Many of the reports are later published, Bledsoe said.

“It’s a pretty good opportunity for undergrads that don’t usually get the opportunity to publish,” he said.

Those students who are looking for a summer program have the option of participating in the environmental field studies program, which places people in national parks throughout the
U.S., including Alaska.

Adric Garlick, a second-year environmental sciences major, worked in Wrangell-St. Elias National Park and Preserve in eastern Alaska.

“The opportunity to travel to some completely wilderness area is incredible in itself,” Garlick said.

Part of his time was spent collecting data on salmon as they swam upstream to spawn. But on his days off, Garlick could explore the Alaskan wilderness in which he was living.

“Some days, I just went on hikes to observe and take pictures,” he said.

Being in Alaska also gave Garlick the opportunity to observe the effects of global warming first hand. He could see how dramatically some glaciers had receded when their current position was compared to pictures from the past.

More short-term opportunities or options for students not willing to travel are hard to come by, according to biology professor Joe Walsh.

“We don’t have an ecology or evolutionary biology department, which is where that stuff happens,” he said.

Students in the past have worked with the Field Museum of Natural History or have conducted projects where they collected data from the Chicago Wilderness nature reserve.

Northwestern does offer a small field ecology class where students visit a forest preserve every week. But things are looking up. In response to growing demand, there are soon going to be more options, Walsh said.

Other field research programs are offered through the Geological Sciences department. Students have the opportunity to participate in spring break trips, such as this year’s visit to west Texas, where students spent time collecting samples to seed an undergraduate research project, said Brad Sageman, the chairman of the geology department.

The department is also planning a trip to Yellowstone National Park for Fall 2006, Sageman said. This option is directed towards undergraduates interested in Earth and Planetary Sciences, a new major currently being developed by the department.
Breaking Down **Attention**

by Nicole Price Fasig

Dr. Marcia Grabowecky is teaching children to control their brain activity. Like rats in a Skinner box, the young minds are learning to respond to rewards and produce higher-level brain waves on command. Got your attention yet?

That is exactly what the Northwestern University psychology researcher and lecturer hopes to study. Grabowecky has spent her career looking at human perception and concentration in the visual environment. She studied psychology at the University of Calgary in Canada where she received her B.A., did her masters work at the University of British Columbia and earned a Ph.D. from the University of California, Berkeley. She completed her post-doctoral fellowship at University of California, Davis’ Center for Neuroscience. Now, she’s working with Professor Satoru Suzuki on two different projects, researching various facets of human attention.

The first is a Herculean effort to build a database of “attention profiles”—800 in all. Grabowecky’s previous research has determined that there is not one all-encompassing form of attention. In fact, various tasks call for different forms of concentration and each individual possesses these faculties in different amounts. Therefore, measuring a person’s ability on various targeted tasks could build a picture of his or her attention profile.

Grabowecky has found that the forms of attention (there are 10 in her study) are not independent of each other, but instead interact in a number of ways. For instance, while some seem to be related, others are mutually exclusive. An individual who is good at central focus, or concentrating intently on one detail or task, will most likely be less capable at distributing his or her attention to a number of stimuli.

Grabowecky does not expect to find one norm; instead she pictures a series of types, demonstrating different combinations of strengths and weaknesses. Individual measures will then be categorized by type and compared to that type’s norm. These new, more complicated measures will account for variation and allow for more targeted studies of individuals.

The project will surely open up new questions in the study of attention types. Another of Grabowecky’s endeavors makes the next intellectual leap: Can individuals control the types of attention?

The project was inspired by a research proposal authored by Katieann Skogsberg, a Northwestern University graduate student. Skogsberg wanted to use neurofeedback to monitor the brain waves of children with Attention Deficit Disorder (ADD) and Attention Deficit Hyperactive Disorder (ADHD). While the idea of teaching children to control their concentration level is not new, little has been done to pursue the matter.

“One of the problems with studying attention is there’s not a lot of basic science
there yet (to show) that it’s an effective theory,” Grabowecy says.

Grabowecy and her colleagues have been holding training sessions for these attention-challenged children, attempting to teach them to control their concentration levels. Children with ADD tend to exhibit more brain waves in the Theta and Alpha ranges; these are characteristic of drowsiness and low-attention levels respectively. Grabowecy says that their brains may have difficulty stimulating neurons to produce waves in the higher, more alert ranges.

In the sessions, the children’s brain waves are monitored via electrodes. Every time the reading enters the Beta range (the next highest above Alpha), the subjects are rewarded with a pleasant chiming noise. The children are initially unsure how to control brain activity, but a sort of operant conditioning works over time and they improve at achieving a certain attention level.

One of Grabowecy’s subjects describes his strategy in a sports metaphor. He pictures the mental state like his mindset when he is getting ready to serve in tennis. He has just thrown the ball in the air and he is on edge, waiting for just the right minute to swing the racket. It is that controlled alertness that the researchers are aiming for, Grabowecy says.

Grabowecy hopes that some day the research may lead to a viable alternative to medication. If children with attention disorders can learn to control their attention levels, they will likely be more successful in school and in life.

“Ideally you’d like kids to be able to work on schoolwork or a book. That’s not like a video game screaming out, ‘Attend to me! Attend to me!’” she says. “Especially when the world’s such a busy, active place, how do you cope with the over stimulation?”

This project is just the beginning. It will lead to more questions and bring up more issues before this form of therapy can be effectively introduced into the market. Grabowecy is looking forward to the challenge.

“People are just fascinating, she says. “I don’t think we’re ever in the foreseeable future going to run out of questions to ask about why people behave the way they do.”
Giving Students Experience Outside the Classroom

by Annie Martin

The Nanoscale Science and Engineering Center (NSEC), headquartered at Northwestern University, provides research internships for undergraduates each year as part of its Research Experience for Undergraduates (REU) program.

The research mission of the NSEC is to create the first tools capable of routinely patterning materials and molecules at the nanoscale (1–100 nm) and to use these tools to make highly sensitive and selective nanoscale sensors. Such sensors could revolutionize many fields including medicine, environmental sciences, and domestic security.

Undergraduates chosen to participate in the NSEC research program engage in interdisciplinary projects with faculty as well as with graduate and postdoctoral associates. The projects focus on advancing discovery and knowledge within the broad field of nanotechnology.

"Every research project has some relevance to other topics," said Vinayak Dravid, professor of materials science and engineering and director of the Northwestern University Atomic- and Nanoscale Characterization Experimental (NUANCE) Center. Dravid further explained, "One student may be working on patterning nanoscale structures, the other may be working on sensing, but they derive some common elements. They are all looking for small-scale structures and looking at the performance behaviors and the basic phenomena of how these materials behave."

The REU program is also offered in the summer and welcomes undergraduate applicants majoring in the sciences or engineering from around the country. The summer program includes nine weeks of full-time research, guest lectures, a field trip to Argonne National Laboratory, interactive public speaking and communications seminars, and special social events. Participants also receive a stipend of $4,500.

"Instead of flipping burgers or mowing lawns during the summer, students are participating in research which prepares them for graduate school, industry, or academia," Dravid said.

Northwestern Researchers were working in the promising field of nanotechnology by the 1990s, and Northwestern has since become recognized as a pioneer in nanotechnology research. In 1999, plans were unveiled for the Center for Nanofabrication and Molecular Self-Assembly (NAMSA) on the Northwestern Evanston-campus. Funded in part by a grant
from the Department of Health & Human Services and private gifts, NAMSA opened in 2002 as one of the first federally and privately funded nanotechnology facilities of its kind in the nation. In 2002, the Institute for Nanotechnology (later to be called the International Institute for Nanotechnology, or IIN) was established at Northwestern.

Many of the accomplishments of IIN researchers—particularly in the development of unique sub-100 nm surface patterning capabilities—laid the foundation for a proposal submitted to the National Science Foundation (NSF) in 2001. This resulted in a ten-year, approximately $25-million award for the establishment of one of the nation’s first Nanoscale Science & Engineering Centers (NS EC s).

“This history of successfully working across disciplines has had an enormous impact on scientific and engineering advances,” remarked Chad A. Mirkin, Director of the NSEC and the IIN, and George B. Rathmann professor of chemistry.

Senior Shaun Elder was accepted into the NSEC for the academic year program in 2006. Although he had been working in a lab, he was looking for a more structured program. Elder began working with Mark Hersam, REU Program Director and professor of materials science and engineering, on a project involving scanning tunneling microscopy and plans to use his research for his senior project in the materials science and engineering department.

“The biggest thing I’ve gotten out of the program is being able to generate significant new knowledge,” Elder explained. “The other big thing is application of stuff we learned in class. We do a lot of theory (in class), but it’s often hard to see how you would actually do a lot of it, so the research has really helped me figure out what the application of it is.”

Junior Gregory Cvetanovich is currently working with Samuel Stupp, professor of materials science and engineering, chemistry, chemical & biological engineering, and medicine, on applying peptide-amphiphiles as a system for encapsulating stem cells and causing differentiation. Cvetanovich started working on this project in 2004 as part of the NSEC academic-year REU program, and has continued his research during the summer of 2005 as part of the summer program.

“It’s really helped me to clarify my future career goals,” said Cvetanovich, who plans to earn a Ph.D. and have a career in research. “It’s helped me realize what research involves, both when you’re an undergrad and a grad student, and see what the professors actually do.”

Cvetanovich and the other group members are performing experiments where, under the right conditions, the peptide-amphiphile nanofibers are able to self-assemble and form a hydra-gel. By cross-linking the peptide-amphiphile nanofibers, they hope to create a stiffer hydra-gel for certain applications such as bone and tissue replacements.

“The undergraduates who have been part of the NSE REU program and worked in my lab have made significant contributions to my group’s research,” remarked Annelise Barron, NSEC researcher and professor of chemical & biological engineering and chemistry. “They have also been an absolute pleasure to work with,” she added.

Additional information about the NSEC REU program is available at www.nsec.northwestern.edu.

With contributions by Kathleen Cook, Director of Operations & Marketing, International Institute for Nanotechnology
Incoming Freshmen Get a Headstart in Science Classes

by Annie Martin

Undergraduate Success in Science (USS) was a summer program for incoming freshman that introduced them to working in a lab and allowed them to acclimate to life at Northwestern before their first quarter even began. Fifteen to twenty-five students were matched with a group of older peer mentors and participated in the program each summer for three summers. The students studied lead levels in soil samples the first two years and stress hormones and biosensors last summer.

Professor Hilary Godwin began the program in 2003 with the intention of retaining more under-represented minority students in General Chemistry although the program was actually open to students of any race. After speaking to students about their experiences in the EXCEL program, which offers incoming McCormick students advanced engineering preparation, Godwin wanted to start a similar program for WCAS students who were interested in pursuing the sciences (though not necessarily chemistry). But rather than starting them early on coursework as in the EXCEL program, Godwin wanted to give them the opportunity to do research. The Howard Hughes Medical Institute, which has a program to introduce research to undergraduates, agreed to fund USS. Participants also completed community outreach projects, which incorporated the knowledge they had acquired throughout the rest of the program.

"It's good for students who haven't done independent research before because it really focuses on teaching them the process of how you get to the point of designing experiments as opposed to just throwing students into a research environment," Godwin explained. "This is a much more thought-out process as to how you lead up to that step and provides you with the skills you need to do independent work."

Veronica Park and Samuel Lee analyze samples of saliva for levels of cortisol and immunoglobulin-A using "biosensor assays" to demonstrate the correlation between stress and immune response. A participant of USS in summer 2005, Park said, "The USS program was an excellent way to meet others interested in research, (including peers, experienced upperclassmen, and professionals) all of this before the school year started. It was an ideal opportunity to ease into the school year."
The program began as part of a nationwide movement toward open-ended, discovery-based labs. The emphasis on learning something new simulates the scientific process much better than simply replicating an experiment in which the answer is already known. In fact, the chemistry department has changed the lab report format to match how real researchers present their data and has been switching over to discovery-based learning labs. Godwin said she thinks this change will better prepare them for independent research and give them a better understanding of the scientific process:

“A large percentage of the students (in USS) will do independent research either during the academic year their freshman year, which is pretty atypical, or go into an REU-type program the summer after their freshman year, which is really unusual because usually the programs don’t take people until they’ve finished their sophomore or junior years. And the reports I get back are that they really do well in those programs—They really go into them knowing what’s expected of them in terms of undergraduate research.”

USS did not end after the summer is over, however. For many participants, their involvement resulted in other research opportunities. Sophomore Mahesh Polavarapu participated in the program as a student in 2004 and then as a mentor the summer after. He also did a continuation of the soil analysis with Godwin during his freshman year, writing a summary of all the students’ USS projects and collecting and testing soil samples from different parks in the Evanston area and informing the community of the lead levels he found in the soil.

“(USS) gives you a good foundation of what they expect from you as far as being a lab student,” Polavarapu said, describing how he benefited. “A lot of kids I know did it because they wanted to get positions in labs their freshman year, so that really helped out because I ended up working for Hilary my freshman year. It got me prepared for college also, because we got a lot of work, and it’s something you’re not used to in high school… Hilary Godwin is the biggest thing that comes out of (USS) because she’s such a great tool to go back to if you need anything.”

On an international level, sophomore Julia Kreger was able to travel to Argentina to work with proteins in a biophysics lab through her work with Godwin.

“I feel a lot of people in the program realize the great connections that they made, and the opportunity really opened up doors for future summers or future research opportunities, whether here at Northwestern or, as I went to Argentina, a different place,” Kreger explained.

Last summer was the end of the USS program because Godwin and the rest of the chemistry department are creating a new chemistry website, finishing the incorporation of biosensors into the program, and developing a separate module for the winter quarter that focuses on nanotechnology and materials science.

“I have totally loved doing the summer program,” Godwin said, reasoning why it was best to stop the program. “It’s been one of my favorite things as a professor. But in terms of reaching the largest number of students and having a permanent impact on undergraduate education at Northwestern, that’s like twenty kids a year versus a thousand in General Chemistry.”
Looking Beyond the Lab

by Angela Chang

Dorothea Koh will be the first to admit she rarely has a class outside of the Tech building. And yes, she does research. But Koh is not a conventional biomedical engineering student. For one thing, she conducts her research in a chemistry lab. And when she applied for a research grant last year, she had some less than usual reasons in mind.

"I wanted to fund my own research so when I screw up I don't have to feel so bad," Koh said.

While most students don't take that into account, Koh, 21, said she often felt bad for using up money from her professor's research grant when she made errors.

But this refreshing attitude is just part of what makes Koh, a junior from Singapore, such a unique researcher. Those that work with her say Koh is a driving force in the lab and puts her curiosity and passion to good use in their current research project, Enhancing DNA Detection. (see page 40)

Koh did not always have any plans to do work in a chemistry lab or to even do research at all. As a freshman, she took Organic Chemistry with Professor SonBinh Nguyen and from there her interest grew. She would stop by Nguyen's office hours and the two would go from talking about problem sets to discussing how material from class could be applied to different things.

"I would give the class a problem and she would always come back with something different and ask me, 'does this work?'" Nguyen said.

Nguyen said Koh's curiosity showed she had something most students do not have.

"She's very passionate about what she's doing and she's curious," he said. "I think those are two things that make a student a good student and make an engineer a scientist."

The class sparked Koh's interest, so she approached Nguyen to ask if he had any research positions open. Nguyen said he thought it was a little strange that she was a biomedical engineering student and wanted to work in a chemistry lab. So he gave her a purely engineering project as her first task.

Koh was assigned to work on an automated pump that sped up the process of coupling DNA with a molecule. Customarily, the reaction was done using two syringes to manually push and pull the fluid, but the process was time consuming. Koh engineered a pump that mechanically pushed the fluid through.
On that first assignment Koh worked with fourth-year graduate student Brian Stepp. The two still work with each other. Stepp, 28, said Koh was notable even as a freshman.

"I was impressed with her ability to rationally think things through," Stepp said. "She came up with key questions we needed to answer."

Since her freshman year, Koh has continued working in Nguyen’s chemistry lab on different aspects of the research project.

In Koh’s research, she wants to be able to tell the difference between one completely complementary DNA duplex and another with one base pair mismatch. Distinguishing between the two can help further the process of detecting cancer and other diseases.

Normally, short DNA duplexes melt into single DNA strands at low temperature and over a broad curve. But the Nguyen lab discovered that if these same DNA single strands are attached to a polymer backbone and allowed to bind to another polymer containing complementary DNA single strands, the resulting hybrid duplexes melt at higher temperatures and with sharper profiles, allowing them to detect the aforementioned single base-pair mismatches. Their findings were published in a paper titled “Organic-DNA Hybrids: Detection Capabilities and Thermal Properties” in Polymer Preprints.

This information can be used to detect cancer or other diseases. Scientists can use cancer genes as a base and apply a patient’s DNA. If the patient has the cancer gene, the DNA will melt at a sharp angle. If the patient does not have the gene, the DNA will melt normally.

But this method had drawbacks. They had no way of placing DNA strands on the polymer uniformly. So there was no way to determine where the DNA strands were attached, how many strands attached or how far apart the strands were spaced.

To remedy this, they switched to a different method. If they used small organic molecules such as benzene instead of polymers, they can tailor them to fix a certain number of DNA strands and also determine how far apart the DNA are spaced, how the strands are angled and various other aspects.

Currently Koh and others in the lab are working on attaching DNA to the small molecules and then performing melting studies to see if these exhibit the same sharp melting curve.

Koh has been working on different aspects of this research project since her freshman year. She credits Nguyen and her advisor, Suzanne Olds, for encouraging her in her research.

“I owe Professor SonBinh everything, and without Professor Olds, I would certainly not have been able to continue working on this interdepartmental project, let alone apply and receive the McCormick Corporate grant. Both of them have been absolutely essential in supporting my research,” Koh said.
Nguyen and Olds were both major supporters when she decided to apply for several undergraduate research grants. Koh recently received funds from several grants and organizations including the Sigma Xi Center, the Sara Boley Undergraduate Research Fund, the American Foundation for Aging Research, the Murphy Society Grant, and the McCormick Corporate Partner Undergraduate Research Grant.

From the McCormick grant, Koh received $5,000 for her research in the Enhancing DNA Detection project. Although the grant funds her research, Koh said her main motivation was so she could financially pay for her mistakes in the lab.

"Every student makes mistakes but she's the only one that feels bad about it," Nguyen said.

Even with all the grants she has been awarded, Koh insists the main reason she receives the grants is because of her letters of recommendation from Nguyen and Olds. In almost all other aspects of her research, Koh shies away from taking too much of the spotlight. She credits others she works with in the lab for making it such a good experience.

"The grad students in my lab are probably the most fun to work with because they're not typical researchers," she said. "People have a stereotype of researchers but no, these people are fun."

Stepp said their lab was unlike most others on campus because the students get along and will hang out even when they're not in the lab.

"When we have group parties at SonBinh's house everybody loves going," he said. "We end up staying until well past the party time and he has to kick us out."

Even with the fun lab atmosphere, Koh is extremely serious about her research. She plans to continue working on the DNA detection project until she graduates this year and hopes to see it succeed.

"She's very passionate about what she's doing and she's curious. I think those are two things that make a student a good student and make an engineer a scientist."

"I'm the kind of person that doesn't like to drop it until I get something out of it," Koh said.

Nguyen noted that her enthusiasm for the research was one of the reasons why they have not killed the project yet. At one point, he said it seemed like they should have stopped. But Koh made a compound that showed the project still had some viability left.

After she graduates from Northwestern University, Koh plans to attend graduate school to study biomedical engineering. Although she likes research and says she enjoys the chemistry aspect, Koh has kept her options open.

One of her other interests is specializing in biotechnology and biomaterials. Koh said she might like to design biosensors or other devices that can be used in medical research.

"I want to work in industry at the forefront of making things and being able to see them change people's lives," she said. "Fundamentally that's what I think I really like about BME, that you can use your engineering skills and be able to make a real difference in the lives of people around you."

Koh, appearing with Nguyen, presented a poster at the Chicago Area Undergraduate Research Symposium in April 2006.
Determining tumor grade non-invasively using quantification of cerebral blood volume through contrast enhanced MRI

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I. BACKGROUND

Determination of relative cerebral blood volume (rCBV) using dynamic susceptibility-weighted contrast-enhanced (DSC) Magnetic Resonance Imaging (MRI) can be used to assess the aggressiveness of a central nervous system (CNS) tumor. One indicator of tumor aggressiveness is vascular proliferation, which leads to increased blood volume. The greater the CBV in the tumor, the more aggressive it is, and the more aggressively it is treated. Current treatments for brain tumors employ a combination of surgical resection, radiation and chemotherapy. In a recent report, Law et al. have shown that if rCBV < 1.75, a tumor is stable with a mean time to progression (tumor growth, increase in neurological deficit or death) of 4620 ± 433 days. If rCBV > 1.75, the tumor is considered progressive with a time of progression of 245 ± 62 days.¹ Law's results demonstrate that MRI has the potential to determine the course of treatment and the response to therapy.

Currently, histopathologic evaluation, or biopsy, is the reference standard for tumor grading; however, this method is limited by tissue sampling error and lack of a specific tumor marker, and it is very invasive.

Background: Central nervous system (CNS) tumor aggressiveness leads to neovascularity and a subsequent increase in cerebral blood volume (CBV). Currently, relative CBV found using Magnetic Resonance Imaging (MRI) can differentiate between stable and progressive tumors, but with high variability and overlap between CBV values of high grade and low grade tumors. We hypothesize that tumor grade can be more accurately determined using a technique we developed to determine quantitative CBV (qCBV). Methods: A series of 18 patients scheduled for routine MRI before surgical resection of CNS tumors were studied. Dynamic susceptibility-weighted contrast-enhanced (DSC) MRI was used to quantify CBV. We compared two complementary quantification techniques: Gradient Echo (GE) and Spin Echo (SE) MRI acquisitions. A region of interest (ROI) analysis was performed to compare qCBV within enhancing regions of the lesion and normal (non-cancerous) tissue. Results: Statistically significant differences in CBV were observed for both acquisitions. GE MRI pulse sequences showed better separation between normal and cancerous tissue and between low grade and high grade tumors; however, GE images were more prone to artifact and field inhomogeneity induced signal dropout near frontal sinuses and auditory canals. qCBV measurements showed a much lower coefficient of variance (CV) as compared to rCBV. This reduced variability confirms our hypothesis, but more data is necessary for a more statistically significant conclusion.
Tumor localization is normally performed preoperatively by contrast-enhanced MR imaging.\textsuperscript{2,3}

While the correlation between rCBV and tumor aggressiveness has been shown, there is significant enough imprecision in the relative measurements of cerebral blood volume (CBV) to make it difficult to distinguish low and high grade tumors. Relative values are determined by comparing the value inside the tumor to the value in white matter (WM), where white matter is the known "control." However, WM Region of Interest (ROI) selection can be imperfect because of a wide range of "normal" CBV, local autoregulation of CBV, or nearby pathology. Furthermore radiation and/or chemotherapy can alter CBV in regions selected as a "control".

A team of researchers in the Departments of Biomedical Engineering and Radiology at Northwestern has developed a means of quantifying CBV by using information that is normally ignored during DSC MRI measurements.\textsuperscript{4,5} Traditional MRI perfusion images provide rCBV by performing a deconvolution analysis on the passage of a bolus of contrast agent. Long after the passage of the contrast bolus, the contrast agent alters the longitudinal relaxation time ($T_1$) of brain parenchyma in proportion to its fractional blood volume. This change in $T_1$, an effect that is normally ignored in MR perfusion scans, is the missing piece of information that allows us to quantify CBV. By making precise calculations of the $T_1$ changes in white matter, we can obtain quantitative CBV (qCBV) over the whole brain.\textsuperscript{6} We hypothesize that quantification of CBV can help decrease the variability in comparison to the relative measurement and create a more definitive distinction between stable and progressive tumors.

**DSC MR Imaging**

All subjects followed the same MRI protocol. A DSC pulse sequence was used to acquire a time series volume of axial slices of the head after contrast injection. DSC imaging tracks a bolus of contrast agent through the volume of interest (VOI). A rapidly injected gadolinium (Gd)-based contrast agent was tracked both through a gradient echo (GE), echo planar imaging (EPI) $T_1$ weighted sequence, and a spin echo (SE)-EPI $T_2$ weighted sequence.\textsuperscript{4} Both GE and SE were used in this study as a means of comparison. While SE pulse sequences generally are not as susceptible to artifact closer to the base of the skull, we used both methods to determine which one was more effective in measuring qCBV.

Using either the GE-EPI or SE-EPI sequence, one can determine the relative concentration of contrast in tissue over time using the changes in signal intensity. The area under this curve is used to find rCBV.

**$T_1$-weighted MR Imaging**

In addition, a time series of $T_1$-weighted images was acquired at a single axial slice. A set of $T_1$ images was acquired both pre- and post-contrast injection. These images were used during post-processing to calculate a patient specific calibration factor to obtain qCBV from rCBV. This scale factor is related to the change in $T_1$ of white matter compared to the change in $T_1$ in blood resulting from the $T_1$-shortening effects of the MRI contrast agent.

**DSC Image Post-processing**

All image post-processing was performed using MATLAB 6.5 R13. The signal intensity of the DSC

![Figure 1. rCBV values for stable and progressive tumors. Notice the variability and the overlap between the two data sets.](image-url)
image is related to concentration by comparing it to the signal intensity before bolus arrival. Thus, using a time series of images, one can solve for the concentration as a function of time, and determine blood volume. The concentration can be found from signal intensity by the following equation:

$$C(t) = \frac{-k \cdot \log \left( \frac{S(t)}{S(t_0)} \right)}{TE}$$  \hspace{1cm} (1)

where \( k \) is a constant, \( S(t) \) is the signal during contrast passage, \( S(t_0) \) is the signal before contrast passage, and \( TE \) is the echo time of the pulse sequence. The signal intensity during contrast injection is related to the signal intensity before by the following expression:

$$S(t) = S(t_0) \left( 1 - e^{-\frac{R(t)}{V_i}} \right) \cdot e^{-\frac{TE}{V_i} \cdot S(t_0)}$$  \hspace{1cm} (2)

Determining the concentration with respect to time can help to determine mean transit time (MTT), CBF, and CBV. CBF is given by

$$k_i C_{Rev}(t) = CBF \cdot R(t) \otimes AIF(t)$$  \hspace{1cm} (3)

where \( C_{Rev} \) is the concentration in the volume of interest, \( R(t) \) is the residue function, \( k_i \) is a constant and \( \otimes \) denotes a convolution. The residue function is the impulse response function for the sample. In other words, it is the characterization of the ideal bolus passage. Thus, convolution with the AIF would give the desired signal. CBV is then related to CBF by

$$CBV = k_i CBF \int R(t) \, dt$$  \hspace{1cm} (4)

The AIF was determined by manually locating the middle cerebral artery (MCA) in the DSC volume and selecting a region of interest (ROI) that would give the curve for the first pass of the bolus of contrast agent. Deconvolution was performed using the Singular Value Deconvolution method as described by Ostergaard, et. al.\textsuperscript{9,10}

**T1 Image Post-processing**

The time series of \( T_1 \) images were first used to create \( T_1 \) image maps using the "lsqcurvefit" function provided by MATLAB with the following equation:

$$S(t) = S(t_0) \left( 1 - e^{-\frac{R(t)}{V_i}} \right)$$  \hspace{1cm} (5)

Curve fitting was performed for the signal intensity of every pixel in the image over the time series to determine \( T_1 \) values stored in a matrix to create a \( T_1 \) map. This was performed for both the pre and post \( T_1 \) images.

The \( T_1 \) of blood was determined by manually selecting an ROI in the sagittal sinus. The \( T_1 \) of white matter was determined by using an automated white matter segmentation algorithm. This is performed by using a histogram of the \( T_1 \) distribution to find the peak representing white matter. All pixels with values in a spectrum determined by this peak were used as white matter.

Using this information, the correction factor was calculated using the following equation:

$$CF = \rho \frac{1/T_{1,\text{post}}^W - 1/T_{1,\text{pre}}^W}{1/T_{1,\text{post}}^\text{blood} - 1/T_{1,\text{pre}}^\text{blood}}$$  \hspace{1cm} (6)

where \( \rho \) is a physiological factor and a constant. In order for this method to work, one must also consider the effects of water-exchange in the brain as described in a recent publication authored by Wanyong Shin MS, a PhD student in the Biomedical Engineering Department. Example images are shown in Figure 2.

![Figure 2](image-url) qCBV color maps for SE and GE pulse sequences with a code for quantification in mL/100 g. The red ring in the posterior right is anomalously high CBV reflecting neovascularity in a confirmed CNS tumor.
**Image Analysis**

CBV images were transferred to a local Syngo workstation for image viewing and ROI selection. ROI selection for white matter and tumor vascular beds were performed on these CBV images by a neuroradiologist with over 20 years of experience in CNS tumor diagnosis. The tumor was localized using an axial T₁ weighted volume after contrast injection. The physician was blinded to the sequence type (SE or GE) and the results of the biopsy.

rCBV was determined by dividing the mean CBV value inside a tumor vascular bed ROI by the mean CBV value inside a normal parietal WM ROI for each patient. qCBV was determined by multiplying the same mean CBV value inside the tumor vascular bed by the calculated correction factor (CF) for quantification.

**III. RESULTS**

**Vessel Enhancement in qCBV Maps**

As shown in Figure 2, tumor aggressiveness leads to vascular proliferation, which causes the ring enhancement around the tumor (red ring) corresponding to an increase in qCBV. With the quantification technique used in this study, ROIs placed in these areas of proliferation can be used to obtain the values for these higher blood volumes.

**qCBV in Lesion vs. White Matter**

Initial results comparing qCBV of lesions and white matter show, in general, a good separation between the two. For the GE pulse sequence, qCBV in WM had a mean of 1.81 ± 0.81 mL/100 g (mean ± standard
Figure 5. Plots showing the values, mean ± standard deviations, and CVs for the qCBV and rCBV of low grade, high grade, and metastatic tumors using GE and SE images.

deviation), while in the vascular area of the lesion, the mean was 8.25 ± 4.80 mL/100 g. The SE pulse sequence showed a mean of 1.72 ± 0.67 mL/100 g for WM and 7.57 ± 5.26 mL/100 g for the lesion.

The GE results seem to show a better separation between qCBV in WM and the lesion because of lower variability in the lesion measurements. This is seen by the higher standard deviation for the SE lesion and the box plots in Figure 3. In addition, the histograms (Figure 4) show that most WM perfusion measurements stay fairly low and around the same values, while the lesion measurements are much more spread out.

qCBV in High Grade vs. Low Grade Tumors

When comparing the quantitative and relative measurements in tumors, the separation between low grade and high grade tumors seems very similar. For GE sequence, the difference between high grade and low grade tumors is about 4.1 mL/100 g for qCBV measurement and 3.56 for rCBV. For the SE sequence, the difference is 1.27 mL/100 g for qCBV and 1.60 for rCBV. Another important result is that the separation between the two types of tumors is much more significant using GE rather than SE.

Despite this similarity between relative and quantitative measurements, the quantitative measurements show a lower coefficient of variance (CV), especially in the case of high-grade tumors. The CV for the rCBV of high-grade tumors is 96.8% for GE and 77.4% for SE. Meanwhile, the CV for the qCBV of high-grade tumors is 80.0% for GE, a 16.8% drop, and 51.2% for SE, a 26.2% drop.

While the data seems similar between rCBV and qCBV for high grade and low grade tumors, the metastatic tumors showed a significant difference between quantitative and relative measurements. As seen in Figure 7, the relative measurements have a much lower mean as well as standard deviation for rCBV measurements in these tumors.
IV. DISCUSSION

Gradient Echo vs. Spin Echo
As mentioned in the Methods section, SE is preferred because of its greater ability to image perfusion near the base of the skull. While this is true, the higher variability measured in the vascularized lesion is of concern. Also, in differentiation for tumor grade, GE seems to produce much better results in terms of obtaining higher values and greater separation for high-grade tumors.

While more data is necessary to discern whether SE data is significantly worse than GE, it may be better to use GE in future studies to ensure less variability, even though we lose flexibility by excluding patients with tumor locations closer to the base of the skull.

qCBV in Lesion vs. White Matter
While there is some overlap between the qCBV values in WM and in the lesion, the results are in general promising. Especially for the GE sequence, the qCBV values tend to be significantly higher in the lesion than in WM. This is an indication that the tumor's vascular proliferation does indeed lead to higher qCBV values.

While it seems that the overlap should be a concern, the major purpose of this study is not to distinguish tumors from WM, but to distinguish tumor grade. Thus, values of qCBV that appear in the same spectrum as the values for WM do not pose a problem as long as they are considered low-grade tumors. Also, some of the higher WM values that overlap with the lower qCBV values may be the effect of radiation therapy on the blood-brain barrier.

qCBV in High Grade vs. Low Grade Tumors
The separation between the mean values of high grade and low grade tumors in relative and quantitative measurement is very similar. However, the lower CV for quantitative measurement indicates that the technique used for determining qCBV in fact decreases the variability of CBV measurements as compared to rCBV. This is especially true in determining the CBV of high grade or progressive tumors, which were the same type that showed such high variability in the Figure 1 from the paper by Law, et al.

There is some concern, however, about specific qCBV values for high grade tumors that were in the range for low grade tumors. While these values may lead to false negatives, according to the data, the larger values will be accurate at predicted true positives. With such a small amount of data at this point, it is not possible to determine a useful specificity or sensitivity. Essentially, while a large qCBV value can be a good indication of a high grade or progressive tumor, a low qCBV value requires further analysis for an accurate conclusion.

The large difference in the mean and standard deviation between the rCBV and qCBV of metastatic tumors are a good indication of the improvement and accuracy that our method provides. This difference could be caused by a higher variability in WM CBV for metastatic tumors. Essentially, patients with metastatic tumors may have a higher physiological variation in normative values. Treatment of the primary tumor may cause the CBV in the WM ROI to be more similar to the CBV in the tumor, causing a lower rCBV value. On the other hand, our technique to quantify CBV eliminates the need to select a WM ROI, thus eliminating the inaccuracies resulting from treatment.

V. CONCLUSIONS
Initial results have proven promising, showing that quantifying CBV improves the CV as well as correcting for error caused by physiological variation in the CBV in white matter. The data has also shown the ability to obtain true positive predictions, which could go a long way in allowing a patient to avoid dangerous neurosurgery. These results indicate some success, but more data is necessary to effectively prove the validity of our hypothesis.

We continue to accrue and analyze patients in the hope of determining a specific value for qCBV in mL/100g, or in other words, a cutoff to distinguish between high-grade and low-grade tumors. While the GE pulse sequence gave better results in this small sample size, we will continue to use both techniques to further explore this conclusion. With the high variability for rCBV measurement, the relation between qCBV and tumor grade should become clearer. Such a non-invasive technique could be a great tool for CNS tumor treatment.
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Evaluation of a Porous Tube Hydroponics System as a Testbed for Remote Sensing Instrumentation

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Accurate detection of plant physiological health indicators such as photosynthetic rates, chlorophyll pigment concentrations, leaf moisture and stomatal conductance are major goals of agriculture, ecological and plant remote sensing research. In this study, we grew Super-Dwarf wheat (Triticum aestivum, cv. Perigee) on a Porous Tube Plant Nutrient Delivery System developed for use in microgravity environments. The system allows control of water availability to the plant root zone by altering the nutrient solution pressures within the porous tubes. Our objective was to assess the potential for detecting differences in indicators of plant health by growing plants at three different root moisture conditions. To evaluate plant health and the potential for utilizing remote sensing to monitor it, we measured percent leaf moisture, plant height growth, photosynthetic rates, photosynthetic quenching, chlorophyll concentrations, fluorescence, and leaf spectral reflectance between 400 and 1000 nm. Our hypothesis was two-fold: first, we hypothesized that plants grown at varying water potentials would exhibit differences in physiological and morphological measures; second, we hypothesized that these differences in physiological measurements would be correlated to spectral reflectance. Differences were detected; plants grown with the most available water had lower photosynthetic rates, greater growth, and higher fluorescence than plants grown with less available water. Significant correlations between reflectance and photosynthesis at a light level of 1000 mmols photons m⁻² s⁻¹ were found in the wavelengths associated with green and yellow visible light along with wavelengths associated with fluorescence.

I. INTRODUCTION

An optimal level of water availability is important for advances in agriculture (i.e., reductions in irrigation expenses and increases in crop yields), fire modeling, and growing plants in space. Plants are expected to exhibit a decrease in photosynthesis and an increase in fluorescence at higher light intensities under water-stressed conditions. Photosynthetic rates measure the rate at which plants convert light energy into chemical energy, while fluorescence is generally the leftover energy from photosynthesis emitted by the chlorophyll. Two regions of the infrared reflectance spectra, the near-(700–1300 nm) and middle-(1300–3000 nm), have been identified as especially useful for the detection of vegetation water stress or water content.³ ⁴ ⁵

Hydroponic systems provide a useful method for examining the effect water availability has upon plant health because they allow for a precision controlled environment. Many advances in technology that control environmental factors such as lighting, nutrients, and atmospheric conditions in the growth environment are facilitating research on plant growth and methods to detect and quantify data using remote sensing instru-
Table 1. Nutrient solution pressures across treatments.

<table>
<thead>
<tr>
<th>Tray</th>
<th>Pressure (cm H₂O)</th>
<th>Potential (kPa)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>10</td>
<td>-0.98</td>
</tr>
<tr>
<td>B</td>
<td>20</td>
<td>-1.96</td>
</tr>
<tr>
<td>C</td>
<td>40</td>
<td>-3.92</td>
</tr>
</tbody>
</table>

Since the nutrient solution pressures are not adequate for optimal plant growth, several changes were implemented. The Porous Tube Plant Nutrient Delivery system (PTPNDS) is a hydroponic system that has been used successfully in the past as a test bed for remote sensing instruments to assess what effect environmental changes have on plant life.

The PTPNDS was developed originally for NASA to grow plants in a microgravity environment. The PTPNDS delivers water and nutrients by pumping them through a porous ceramic filter tube at a defined nutrient solution pressure so that the plant roots can uptake them through capillary action on a moistened ceramic surface (Figure 1a).² By using this system to test remote sensing instrumentation and examine variables such as photosynthetic rate, stomatal conductance, fluorescence, and reflectance as measures of plant health, a precedent could be set for future implementation into systems and protocols. These systems and protocols would be greatly beneficial to manned space exploration and terrestrial based applications such as precision farming and land management. Plants that are photosynthetically healthy fluoresce less energy given a unit quanta of light and could have more chlorophyll pigments in the leaf tissue. Wavelength dependent reflectance measurements should respond to these changes in chlorophyll concentrations and fluorescence rates.

Figure 1. Diagram of the Porous Tube Plant Nutrient Delivery system. Schematic of whole system (a);³ operational diagram of porous tube function (b).⁴
II. OBJECTIVES OF INVESTIGATION

Our main objective was to determine if indicators of plant stress are detectable when plants are grown with small differences in water availability. We expected plants grown under a more negative nutrient solution pressure would exhibit physiology associated with water stress. Our second objective was to determine whether differences in the indicators of plant stress (photosynthesis, fluorescence, moisture content, and chlorophyll concentration) could be correlated to reflectance.

III. METHODS & MATERIALS

In this study, wheat plants (Triticum aestivum, cv. Perigee) were grown on three trays (A, B, C) with eight porous ceramic tubes each. Each tray (Table 1) had a different nutrient solution pressure. The plants were grown on a PTPNDS developed for use in microgravity environments. We varied nutrient solution pressure and held other variables constant by changing only the standpipe height. Percent moisture, chlorophyll content, and other aforementioned stress variables were measured.

- A bi-weekly log of plant heights (Figure 2) was kept along with humidity, temperature, and light intensity readings in order to analyze them statistically. Measurements were taken starting 6 days after planting (DAP).
- At 21 DAP, six leaves from each tray were harvested, weighed, analyzed for reflectance spectra using the ASD FieldSpec Pro, and put in a drying oven for 48 hours to determine percent moisture loss.
- At 21 and 22 DAP three leaves from each tray were harvested, ASD spectra read, and chlorophyll extracted in a DMSO-Acetone solution for twenty-four hours. Subsequent chlorophyll pigment concentration analysis and reflectance spectra were collected using the Beckman DU-640 Spectrophotometer.
- Also at 21 and 22 DAP three leaves were analyzed live and in situ for fluorescence and photosynthetic rates using the LI-COR 6400 Portable Photosynthetic System. They were subsequently harvested, measured for mass and area, ASD spectra read, and chlorophyll extracted and analyzed.
Data were analyzed using SPSS statistical software and graphed using SigmaPlot 2001 and Microsoft Excel.

IV. RESULTS
Height growth was similar between all three trays for the first 20 DAP. However Tray C, with the least available water, had significantly less height growth (p < 0.001) from DAP 25 on (Figure 3).
Percent leaf moisture was highest for plants grown on Tray A, which had the most available water and lowest for plants grown on Tray C which had the least available water (Figure 4).
Differences in physiological parameters were detected between plants grown on different trays. The plants grown with the least amount of available water, Tray C, exhibited the highest photosynthetic rates (Figure 5a) and the highest light harvesting efficiency (Figure 5c). Plants grown with the most available water, Tray A, did not differ in photosynthesis from plants grown on Tray B; however, they did exhibit the highest levels of fluorescence as seen in the larger proportion of non-photochemical quenching (Figure 5b).
Differences in mean reflectance signatures were detected for the three treatments (Figure 6). Minimum reflectance values occurred in the blue (400–525 nm) and red (600–675 nm) wavelength regions with higher values in the green and near infrared. Significant negative correlations were found between LI-COR measured photosynthesis and wavelength specific reflectance at the light level of 1000 mmols photons m⁻² s⁻¹ (Figure 7). These negative correlations were observed in the green and yellow (500–625 nm) wavelength region and in the photon fluorescence region at approximately 690–720 nm.
Chlorophyll A concentrations ranged between 1.03 and 2.48 mg/g wet weight. There were no discernable differences between treatments and no significant correlations were found between reflectance and chlorophyll concentrations.

V. DISCUSSION & CONCLUSIONS
Differences in physiology and morphology were found between plants grown at varying water availabilities. Counter to our expectations the plants grown with the most available water were the plants that exhibited the most signs of stress, having lower photosynthetic rates and higher fluorescence. However, the plants grown with the least available water did show slower growth as the plants aged. The plants grown on Tray C were not only visibly greener, but also had higher photosynthetic rates. The reflectance spectra (Figure 6) showed that the plants on Tray C absorbed more available light along the entire visible light spectrum, with noticeable differences occurring in the 500–700 nm (the green, yellow, red range). This difference was also present at the wavelength in which fluorescence is detectable (≈715 nm). The significant negative correlations between photosynthesis and reflectance suggest it is possible to monitor small differences in plant health indicators using remote sensing technology. These data indicate two spectral regions may be used, the green-yellow and the red fluorescence wavelengths.
VI. FUTURE WORKS

Future research will focus on controlling peripheral variables such as light intensity distribution, constant nutrient flow, and atmospheric controls in order to be better able to test sensitive experimental parameters such as chlorophyll pigments and their relationships to other plant stress and health factors.

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Biomedical Implants:
The fabrication and reliability testing of silicone encapsulated, non-hermetic devices

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I. INTRODUCTION

During the past few years, biomedical implants have revolutionized the world of healthcare, technology, and engineering. Biomedical implants will enable people with various disabilities to lead more normal lives and reduce their health risks. Until recently, most of these devices have been sealed in metallic, non-flexible hermetic packages. These hermetic packages present several problems due to their cost and size. Recent trends have begun to shift away from these metallic hermetic implants towards soft biological polymers, such as silicone. Silicone hermetic implants are used to encapsulate both active and passive electrical components, which are very dependent on changes to the external environment. It is therefore necessary to determine if these new implants will be capable of functioning inside the human body for several years without malfunctioning.

To determine this, a test chip had to be created. The chip mimicked an implant aimed at helping persons with spinal cord injuries or those who have suffered a stroke. The goal was to create a “functional electrical stimulation” (FES) system that stimulates nerves in the central nervous system that are activated at lower electrical charges than muscles.

Various properties of silicone make it a good candidate for encapsulation of such biomedical devices. Silicone adheres to a variety of materials and forms hydro-thermally stable joints. It is soft and does not produce large shrinkage problems or void formation. Also, it is impermeable to Na⁺ and Cl⁻ ions. Despite some concerns that since silicone membranes are permeable, liquid water will be able to seep through the material and destroy the electrical equipment inside the implant; it appears that this will not occur when placed inside the human body for osmotic reasons. Note that silicone is also penetrable to water vapors, which may fill voids in the silicone and destroy the imbedded electrical components. Therefore, void-free encapsulation is integral to the success of the silicone implants.

The aim of this work was to create a test chip, encapsulate it in silicone, and place it in an environment similar to the human body. Rather than being a direct representation of the actual implant chip, the test structure was used to assess the process of fabrication and the effects of assembly design. The chip was submerged in a saline solution to mimic internal human body conditions. A constant bias of 10V was applied to it over a period of six weeks to accelerate its failure. In addition, the test structure could be used to monitor failure mechanisms such as electro migration (short circuit) and corrosion (open circuit).
II. METHODS

A. Substrate Creation

The test chip used a ceramic substrate consisting of 96% aluminum and 4% silica. Two gold track patterns separated by a blue glass dielectric were created in the pattern of a meander. Two of the tracks ran above the dielectric while the other two ran below it. Each chip also had 8 gold pads, to which the wires were soldered. The implants were highly susceptible to corrosion and it was important to choose metals that demonstrated a level of corrosive resistance. Gold was chosen for this project because it was easiest to manipulate mechanically. The PCB substrate is demonstrated in Figure 1.

This test structure is well suited for reliability testing, as it is easy to manufacture and is very similar to the actual chips. Reliability testing involved applying a power source to the chip and monitoring how long it lasted without malfunctioning. The first step was to attach copper wires using a soldering iron. The optimal temperature for the soldering of copper wires onto gold was 450 degrees Celsius, which is much higher than for other metals. The strength of the joints was tested through the use of an ohmmeter, connected to the end of the wires. Figure 2 depicts the solder joint.

Figure 1. A ceramic PCB plate that was used in the design of the silicone encapsulated test chip. Two of the gold tracks are visible above the blue glass dielectric connected to the gold pads. This is before the application of silicone.

Figure 2. A solder joint between the copper wires and the gold pads on the PCB test plate.

Figure 3. The silicone encapsulant mold without the test plate. The picture shows an empty mold before the application of silicone. The PCB test plate would be placed in the middle with the copper wires protruding through specially made holes on the side.

Figure 4. The silicone-encapsulated sample shown after it has cured. This shows the completed product after the attachment of the copper wires and the removal of the test plate from the mold.

B. Encapsulation

In order to encapsulate ceramic test plate in silicone, a plastic mold was created. The ceramic plate was placed inside the mold and the soldered wires protruded from specifically designed holes in the mold. The silicone was dispensed from a pressurized syringe at a pressure of 20 psi into the mold. The mold is demonstrated in Figure 3.

The 20 psi pressure was necessary to eliminate the number of air bubbles that could develop into voids, attracting water vapor. The sample was left overnight in a hood in order to cure. As the sample cured, it absorbed moisture from the surrounding atmosphere thereby hardening and transitioning from a semi-liquid viscous state into a solid one. After it cured, the silicone sample was removed from the mold with the use of a scalpel. The final product is demonstrated in Figure 4.

C. Reliability Testing

During the testing stage, two main sources of failure were monitored: electro migration and corrosion. Electro migration would create voids in a conductor that could cause connection of meander tracks. An indication that electro migration had occurred would
result in a sharp decrease of the monitored resistance values. The second potential failure was corrosion, in which circuit components would degrade, thus creating a sharp rise in the resistance measurements.

In order to monitor these two failures, power was applied to the sample and resistance values were measured periodically. This was done through a multiplexer with five leads applied to the sample.

The sample was placed in a vial containing saline solution. The vial was sealed and placed in a water bath with the temperature around 37 degrees Celsius.

Six samples were tested in this experiment. Samples 1 and 2 were created at Tyndall National Institute and were nearly identical except for one minor, but important issue — Sample 1 had contained a drop of epoxy glue between the bottom of the chip and the silicone thin sheet, whereas Sample 2 had not. A partner company involved with implant manufacturing assembled Samples 3-6. Samples 3-6 had several different parameters including the number of dielectric layers, the type of component adhesion, the quality of the encapsulation process, the cleaning process of the sample, and whether the encapsulation had been controlled. Samples 3-6 were tested against Samples 1 and 2 to see how long different samples would last and whether the parameters described earlier had an effect on their lifespan. The six samples were tested over the course of six weeks. Readings were taken every day at thirty-minute intervals.

III. RESULTS AND ANALYSIS

For the first four weeks, all the samples had lasted with no visible changes to their appearance or a rise or drop in resistance values. All the resistances had values between 1.6 and 2.0 ohms. However, during the fifth week of testing, readings for the Sample 2 rose from 1.83 ohms to 0.31 mega ohms. During the final week of testing, Sample 1 was visually inspected and seen to be failing.

The sudden rise in the resistance for Sample 2 at first seemed indicative of corrosion. When the sample was taken out of the saline solution and analyzed, a large void cavity surrounding the joint was discovered that had indicated the resistance rise. Also, the copper wire that was partially exposed to the formed void was completely eroded away, and instead a bright blue residue had appeared. The residue had been indicative of oxidation, and the cause of failure seemed to be water moisture that had crept into the void combined with a constant bias of 10 Volts. There was no longer a proper contact in the meander, and it was concluded that the implant had failed due to electrical corrosion. The joint was also analyzed using a metallurgical microscope and appeared to be brittle and damaged. There are several possible explanations for the failure of this device.

The bottom of the Sample 2 had not been glued to the silicone thin sheet. When the sample was turned over and analyzed, there was a thin layer of water trapped in the hollow space. This is one of the

<table>
<thead>
<tr>
<th>Sample</th>
<th>Corrosion Defects Observed</th>
<th>Electro migration Defects Observed</th>
<th>Final Resistance Values (Ohm)</th>
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</thead>
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<tr>
<td>1</td>
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</tr>
<tr>
<td>2</td>
<td>Yes</td>
<td>No</td>
<td>310000</td>
</tr>
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</tr>
<tr>
<td>6</td>
<td>No</td>
<td>No</td>
<td>1.80</td>
</tr>
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</table>
potential routes that the water took to seep in and ruin the implant. The second and more probable route had been the void that originated around the joint. When the silicone in this sample had cured, there were several air bubbles next to the PCB plate. One of these bubbles had been close to the solder joint that failed, so a logical conclusion would be that it had allowed moisture to creep in and expand the bubble, creating a damaging void.

When Sample 1 was taken out of the saline solution and visually analyzed, there appeared to be a wearing down of the meander track. This had occurred around the bends of the meander and was very noticeable in the upper right corner of the chip. Also, the same bright blue oxidative residue was visible. These two factors create the impression that the sample had also failed due to corrosion as the circuitry was eaten away. However, it is interesting to note that although upon visual inspection these problems were clear, they had no affects on the resistance readings. This is due to the fact that failure had not yet achieved its optimal destruction and would still damage the chip more before the resistance values were affected. The failure of Sample 2 is probably due to the fact that the back of the chip had not been glued down. Also, when the silicone covering was applied, it was applied at a higher pressure of 30 psi, making the silicone cover the chip faster, but also allowing more air bubbles to form. The performance results of all samples are summarized in Table 1.

IV. CONCLUSIONS
The goal of this project was to create a silicone-encapsulated test chip similar to those used in the biomedical industry and test it over a period of six weeks in a near-body environment using a constant bias to accelerate failure. The fact that only one of the six test samples failed over the course of testing period demonstrates that silicone encapsulated implants can become successful biomedical devices. However, there are still several improvements that could be done in the future. One such improvement could be redesigning the mold. Currently, the mold is designed so that the holes are only small enough for the wires to come out of it. It can be improved by making the holes larger. This will allow a small amount of silicone to fill the holes and spread along the length of the wire, creating a small airtight sleeve.

Despite the necessary future improvements, silicone-encapsulated implants are a major breakthrough in the field of biomedical engineering. Whether correcting cardiac arrhythmia or sending impulses to stimulate peltal nerves and muscles, these implants have the potential of revolutionizing healthcare and patient comfort. If successful, they will eliminate the staggering costs and patient discomfort associated with metallic implants.

Acknowledgements
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REFERENCES
On-Line vs. Off-line Quality Control Systems: A Comparison of Taguchi’s Method to a Control Chart-based Approach

Taguchi’s on-line quality control method is compared to a hypothetical off-line quality control method consisting of the use of X-bar and R charts; this is done for a specific case of a Motor Company’s cylinder block production line. The means of comparison is the cost of quality control per unit produced as reflected by Taguchi’s Loss Function for the on-line method and an analogous function for the off-line method. This paper proves that, as proposed by Taguchi, the use of an on-line quality control method—in this case Taguchi’s Diagnosis and Adjustment of Processes—is a better option for the specific process of cylinder block hole-bearing within the production of engines. However, it is realized that the application of on-line or off-line quality control systems should always be considered since the degree of specificity of each manufacturing process is high. Therefore, the use of off-line quality control systems should not be disregarded.

I. INTRODUCTION

Genichi Taguchi, well-known statistician and engineer, has done extensive work on the development of manufacturing quality control systems. In his chapter “On-Line Quality Control System Designs,” found in Park and Vining’s Statistical Process Monitoring and Optimization. Taguchi presents two methods of reducing the cost of “controlling quality” in a manufacturing process. Before presenting his methods, Taguchi assures that on-line quality control systems are a superior option to off-line quality control systems in keeping the production lines from falling out of control; however, he does not present proof of this. It is the purpose of this paper to verify the higher effectiveness of Taguchi’s on-line quality control system in comparison to a more old-fashioned off-line quality control system.

Taguchi’s Diagnosis and Adjustment of Processes, an on-line quality control method, is compared to a hypothesized off-line quality control method. In this case we use an X-bar control chart; additionally the case of an R-chart used in combination with the X-bar chart is also considered.

Due to the importance of cost in production processes, and to the availability of literature describing the application of Taguchi’s system to a specific process, the means of comparison between the two methods includes the analysis of loss functions, which assess the quality control cost per unit produced in each of the two quality control systems.

II. THEORETICAL BACKGROUND

On-line and Off-line quality control systems

During the 1920s, Walter A. Shewhart, a physicist working for Bell Laboratories, saw the necessity of the creation of some type of method to monitor the quality of the products coming out of a manufacturing process. He used the tools of statistics to create what, today, we call Shewhart control charts, which are described in this section. The main purpose of these charts is to
serve as tools for detecting irregularities in a process. This enables engineers to find "assignable causes" for these irregularities, make the appropriate adjustments in the process, and try to prevent the occurrence of these causes in the future—this is called off-line quality control. Taguchi names the alternate to off-line quality control on-line quality control, "the routine quality control activity on production lines." This method, instead of using charts to analyze data before taking action to correct any irregularities in a process, tells the process engineer to take action as soon as a certain attribute is given to a single product unit in the process.

**Taguchi's Method**

In "On-Line Quality Control System Designs," Taguchi describes a method (Diagnosis and Adjustment of Processes) in which an output unit characteristic (such as length, weight, etc.) in the process is measured every \( n \) output units. The characteristic is then compared to a specification range, and, if the unit shows a deviation greater than a certain specification limit, it is discarded and the process is stopped. The rest of the items being produced are assumed to come out with the same, or still "unsatisfactory," characteristic and so necessary action is taken in order to bring the process back to normal, in-control, production—stage at which production can be continued. Taguchi modeled a Loss Function, in which he considers all the costs incurred by following this type of on-line quality control. He argues that this type of quality control is the way to keep a process under control running smoothly, compared to off-line quality control systems. From this description, it is evident that this method does not include a broad application of statistics techniques; however, some probability comes into play in the description of Taguchi's Loss Function. This Loss Function will be analogized in this paper as a means of comparison between Taguchi's method and a hypothetical off-line method using control charts.

**Control Charts**

As mentioned above, Shewhart invented the so-called control charts as a tool that would allow engineers, managers, and machine operators to monitor and control the output of industrial processes. Shewhart charts (as they are also called) assume that any process output has two types of variability. The first one is called noise and is due to complex causes such as the slow deterioration of machines, dust, varying temperature, etc. The second one is due to an "assignable cause," which can go from the lack of experience of new machine operators to the malfunction of a machine part. As John explains, Shewhart charts are built under the assumption that the built-in variability of a process is completely random and shows a certain amount of regularity, thus being normally distributed.

Shewhart proposed that processes should be monitored by taking samples of four or five output units at regular intervals and making appropriate measurements of a certain characteristic—such as length—to those samples. Two plots are then constructed from the acquired data, one showing the sample mean, X-bar, of the length measurements, and another showing the sample variability, represented by the sample range. In both charts, the acquired data is compared to a target mean and "acceptable" variability of the process, which are measured from a control group of output samples (usually about 20) taken during a period in which the process is assured to be working in optimum conditions.

**The X-bar chart**

The X-bar values are plotted and assumed to have a normal distribution with mean \( \mu \) and standard deviation \( \sigma/\sqrt{n} \), where \( \mu \) and \( \sigma \) are the mean and standard deviation of the control group taken as pooled data and \( n \) is the number of units in each sample, respectively. It can be shown by statistical methods that the mean of the variable X-bar is equal to the mean of the variable \( X \), for each single unit, and that the standard deviation for X-bar is equal to the standard deviation of the variable X, for each unit, divided by the square root of the number of units in each sample.

The X-bar values are plotted versus the sample number and horizontal lines are drawn at \( X \)-bar = \( \mu \), \( \mu + 3\sigma/\sqrt{n} \) (the upper control limit, UCL), and \( \mu - 3\sigma/\sqrt{n} \) (the lower control limit, LCL). Due to the normal distribution of X-bar, the probability that it lies above or below the \( \mu \pm 3\sigma/\sqrt{n} \) range is 0.266. Adding up the probabilities of subsequent units failing in this range until the sum is 1, one can see this will happen, on average, once every 1/0.0026, or 385, units. Since the probability of a point in the X-bar plot falling outside of the control limits due to more chance is so low, the reason for their being chosen, a process is said to be "out of control" whenever this happens.
This process is then stopped, and the operators and engineers proceed to look for an "assignable cause" for the unexpected deviation. After the assignable cause is found, it is fixed and then production is continued. If no problem is found, production is simply restarted.

There are many other ways in which an X-bar chart can help monitor a process and it is up to its user to set the rules for stopping this process and looking for an assignable cause. For instance, if one sees something like ten points lying above the μ line, one may speculate that the process's noise is not behaving randomly and may thus decide to stop it and check if there is something wrong with it. Or, if one sees two or three points lying very close to the UCL or LCL, then one may also conclude that something may be wrong and decide to stop the process. Some engineers also draw two lines at μ ± 2σ/√(n) as "warning" control lines, outside which only 4.56% of the values will randomly lie. These limits are helpful in creating new "warning" rules, which can be used as signals to check the process, without the necessity of completely stopping it. This last warning feature is an important advantage of the use of control charts over Taguchi's on-line control system.

The R chart

The R (Range) chart helps the monitoring of the variability of a process's output. In it, the ranges (largest value - smallest value) of each sample are plotted and considered to be random variables with normal distribution, just as done in the case of the X-bar chart. The target value for this chart, μₗ, is the mean of the ranges obtained from the samples in the same control group used to obtain the reference values for the X-bar chart. The upper and lower limits are found by

\[ UCL = \mu_r + 3 \left( \frac{d_2}{d_3} \right) \mu_r \]
\[ LCL = \mu_r - 3 \left( \frac{d_2}{d_3} \right) \mu_r \]

where \( d_2 \) and \( d_3 \) are constants that depend on the sample size and can be found in statistical quality control tables. By analogy to the X-bar chart, one can see that the value \( \left( \frac{d_2}{d_3} \right) \mu_r \) is an estimation of the standard deviation of the range values, which is convenient for determining the limits of a normally distributed variable. Once more, the probability that a value plotted in this chart will lie outside the control limits is therefore 0.266%. It is useful to have an R chart since the X-bar chart by itself could be misleading if, for instance, a process had samples with large ranges but

![X-bar Chart](https://example.com/xbar.png)

**Figure 1. X-bar Chart (each point refers to a different sample).**

Hypothesis Testing

As applied to the field of quality control and, more specifically, to sampling techniques, the test consists of two hypotheses.

For the case of an X-bar chart the two hypotheses are:

\[ H_0: \text{ "process is out of control" (null hypothesis) } \]
\[ H_1: \text{ "process is in control" } \]

Whenever the null hypothesis is not accepted, the alternative hypothesis, \( H_1 \), is automatically accepted. In addition, a Type I error is committed if the null hypothesis is accepted when it is not true and a Type II error is committed if the null hypothesis is rejected when it is actually true. Furthermore, there is a probability \( \alpha \) of committing a Type I error and a probability \( \beta \) of committing a Type II error. In the case of an X-bar and R charts, where the limits are controlled by three standard deviations, \( \alpha = 0.0026 \).

Process Capability

As defined by Burr, the Process Capability

\[ C_p = \frac{US - LS}{6\sigma} \]

where US and LS are the Upper and Lower Specification limits of the characteristic in treatment and \( \sigma \) is the estimated value of the standard deviation of the process in treatment, found from the above mentioned control group. It is important to clarify here that US and LS do not necessarily have to be the same as the UCL and LCL. The specification limits are the ones that have to be met in order to satisfy product specifications, while the UCL and LCL are limits that are set by the natural noise of the process - this natural noise determines the process's capability. As we can see from the definition of
$C_p$, a process with $C_p = 1$ is one that produces all units under specification. Today, many Japanese companies use $C_p = 1.33$, which means that all products are made under specification and that, if the process goes out of control, some units produced during this stage may still be under specification.\textsuperscript{12}

### III. Taguchi’s On-Line Quality Control System vs. A Hypothesized Off-Line Quality Control System

As mentioned in the introduction, Taguchi argues that an on-line quality control system is a better way of keeping a process in control. This will be verified in the following way:

In “On-Line Quality Control System Designs,” Taguchi presents an application of his system to the specific case of the cylinder block production line of a Motor Company in the 1970s. He then presents a Loss Function, which he uses to assess the quality control cost and to prove that his derived optimum measuring interval size is correct.\textsuperscript{13}

For the profit-making purpose of most manufacturing plants, the cost of the quality control system that the plant is utilizing is the heaviest criterion considered in deciding which quality control method to use, provided that all alternative methods attain the same goal. In the comparison of Taguchi’s on-line method to a hypothetical off-line method applied to the same production line, both systems attain the same goal, therefore, the basis of comparison in this paper will be the cost of applying each system. This leads to the creation of a loss function, analogous to Taguchi’s, for the off-line system as well.

From this point on, the following case proposed by Taguchi\textsuperscript{14} is considered.

#### I. Motor Company’s Cylinder Block Production Line

Let us picture a production line of cylinder blocks that have some specific purpose within an engine. One of the steps in this production line is the boring of holes by reamers. Ten holes are bored at a time in each cylinder block. For simplicity, suppose that the target position of the holes is $\mu$. If the holes are misaligned by more than 10 $\mu$m, then they will not be accepted by the client and are thus useless. Therefore, the specification limits of this process characteristic – the alignment of the holes – are $\mu \pm 10$.

Taguchi’s approach to quality control in this case

Taguchi’s proposed method is that holes are measured every $n = 16$ produced cylinder blocks (units). The cost of checking or diagnosing the process each 16 units is 400 yen, denoted by $B$. A cylinder block is scrapped as defective if there is any hole bore that is misaligned by more than 10 $\mu$m, causing an 8000 yen loss, denoted by $A$. When there is a problem such as a crooked hole, the production is stopped, the reamers are replaced, the first hole bored after the replacement is checked, and if it is normal, the production is continued. The time lapsing – measured in units produced – between the moment a problem is found (i.e. holes are misaligned by more than 10 $\mu$m or holes are crooked) and the moment when the process is stopped, called the time lag, is denoted by $t$, and is equal to 1 (on average). The total cost, including the cost of stopping the production line, tool replacement, and labor, is called the adjustment cost and is equal to 20,000 yen, denoted by $C$. Although Taguchi does not mention it in his article appearing in Park and Vining, Nayebpour and Woodall\textsuperscript{15} affirm that this cost also includes the “retrospective inspection cost,” which is the cost incurred if one decides to find defective items amongst the above-mentioned measuring interval of $n$ units. For the purposes of this paper, this cost will be taken into account and assumed to be included in $C$.

In a period of six months of work for the process, 18,000 units were produced and seven quality control problems (in which all costs are incurred) were found. Therefore, the average problem occurrence interval is $18,000 / 7 = 2570$ (units), denoted by $u$.

It is worth noting that, although Taguchi does not mention it in the article,\textsuperscript{16} his on-line quality control approach assumes that all units produced after a defective unit are also defective, as confirmed by Nayebpour and Woodall.\textsuperscript{17}

Taguchi’s Loss Function, which gives the quality control cost per unit produced, is:

$$L = \frac{B}{n} + \frac{1}{2} \left(\frac{(n+1)A}{u} + \frac{C}{u} + \frac{tA}{u}\right)$$

The first and third terms give the diagnosing and adjustment costs per unit produced, respectively. $B$ is divided by $n$ since $B$ is incurred every $n$ units, when a measurement is made. $C$ is divided by $u$ since $C$ is incurred every time a problem occurs and this happens...
every \( n \) units. The last term accounts for the cost of producing defective units after it has been noticed that the process has a problem; this, again, occurs every \( n \) units. The second term accounts for the loss per unit produced due to the production of defective units during the measuring interval \( n \). Taguchi proposes that the average number of defective units is

\[
\frac{1}{2} (n + 1)
\]

This argument assumes that the probability of a unit having a problem is the same for all units produced within a measurement interval.\(^{15}\)

For the process just described, Taguchi’s Loss Function equals:

\[
62.4 \text{ yen/unit produced}
\]

**Hypothesized Off-Line Quality Control System**

The case of the use of a single X-bar chart applied to the same exact process is treated. Let \( X \) be the alignment position of the holes bored in the cylinder blocks.

One may suppose that the process has been running long enough so that the variable \( X \) is normally distributed with average \( \mu \) and standard deviation \( \sigma \); these statistics are used instead of the usual control group.

Furthermore, suppose that this process has a \( C_p \) value of 1 (as is usual for companies in the United States), which means that \( 3\sigma = 10 \mu \). Suppose also that every time the process is stopped, things are corrected in such a way that \( \mu = \mu_0 \) so that the process is under specification as long as it is under control. With these assumptions, one can see that, under Taguchi’s method, the process is stopped every time \( \mu + 10 > \mu_0 + 3\sigma \) or \( \mu - 10 > \mu_0 - 3\sigma \).

In the hypothesized off-line system, instead of measuring a unit every \( n \) units, a sample consisting of \( m = 5 \) units will be measured every \( N \) units – the optimum value of \( N \) will have to be found – and the average will be found and denoted X-bar. The variable X-bar will be normally distributed with average \( \mu \) and standard deviation \( \sigma/\sqrt{m} \). The X-bar values will be plotted in a classic X-bar chart, which will serve as a basis for deciding to stop the process or not; this will be done every time X-bar > \( \mu + 3\sigma/\sqrt{m} \) or X-bar < \( \mu - \sigma/\sqrt{m} \), which gives an “out-of-control” signal. One can see that this will happen every time the process is stopped under Taguchi’s on-line method so, for this method, the average problem occurrence interval will be \( v = n = 2570 \) units.

**Loss Function for the Off-Line Quality Control Process**

In order to create a function that is comparable to Taguchi’s Loss Function, careful analysis of the hypothesized process is needed. A term-by-term analogy follows.

First Term: \( B/n \)

This term represents the diagnosis cost per unit produced. In Taguchi’s function, \( B \) is the cost for diagnosing one unit. For the new function, let \( \beta = B \), since the cost of diagnosing one unit will not change. Since in the off-line system \( m \) units will be measured, this diagnosis cost will go up each time the production line is stopped to take \( m \) measurements. However it will not be directly proportional since measuring additional units should not be as costly as the first unit measured. So this term may be substituted by:

\[
\frac{\beta + .8 (m-1) \beta}{N}
\]

Second Term: \( \left( (n+1)/2 \right) \cdot (A/n) \)

It is satisfactory to assume that the probability of a unit being defective will not change by the change of the quality control method. Furthermore, the cost of producing a defective unit, \( A \), will also be the same. Letting \( \alpha = A \), this term will be:

\[
\frac{1}{2} \frac{(N+1) \alpha}{n}
\]

for the off-line function.

Third term: \( C/u \)

Let \( \pi \) be the adjustment cost for this off-line function. This time, the adjustment cost will be different from the one in Taguchi’s function since it includes the retrospective cost of searching for defective units. Since in this process \( N \neq n \), this searching cost will be different. Supposing that the cost of searching for the defective units is 30% of the total adjustment cost, the value of \( \pi \) modified for searching over \( (N - (m-1)) \) units and for \( m = 5 \), follows:

\[
\pi = .7C + \frac{.3 (N-4) C}{n}
\]

where \( n \) is the interval size in Taguchi’s function. Thirty percent is subtracted from \( C \), weighted by the factor \( (N-4)/n \), which accounts for searching over more units than \( n \), and then added back to the total cost for this term. The third term becomes:
Fourth term: \( \frac{\pi}{v} \)

Assuming the same amount of time lapses between noticing that the process is out of control and stopping the process in using both methods, let \( \delta = l \), and the fourth term becomes:

\[
\frac{\delta \alpha}{v}
\]

which is identical to the fourth term in Taguchi's function.

More terms need to be added to the off-line control loss function to account for several differences in the quality control process.

In the first place, one should note that, by using an X-bar chart in which the process will be stopped every time a point lies outside the control limits (plus or minus three standard deviations), a Type I error will occur. This means that the process will be falsely stopped every 385 units. In doing this, only a fraction of the total adjustment cost will be incurred, namely the costs of not producing some units and of checking that there are no problems with the process. Suppose that the fraction of the total adjustment cost is 20% and let \( \text{Ifactor} = 1/385 \) be the variable that denotes the frequency of committing a Type I error. The new extra term is:

\[
- \frac{.8 \text{Ifactor} \pi}{v}
\]

which implies that 80% of the total adjustment cost will be saved every time a Type I error is committed.

Another "saved" cost occurs every time a Type I error occurs will be the cost of the units produced during the time lag \( \delta \), in which defective units are produced. If the process is stopped and found to be working fine, then no cost is incurred for producing defective units before the process is stopped. This new saving term is:

\[
- \frac{\text{Ifactor} \delta \alpha}{v}
\]

In the case of committing a Type II error, which is the failure to stop the process when it should have been stopped, no extra cost will be incurred. This is because all the interval units are being checked every time an out-of-control signal is received at a sampling point.

Furthermore, the cost of checking them is already included in \( \pi \) and the cost of producing these defective units is accounted for in the \((N+1)/2\) factor of the second term in the function.

Putting the terms together, the loss function for the hypothesized off-line quality control, \( \lambda \), is:

\[
\lambda = \beta + .8(m-1)\beta \frac{1}{N} \alpha + \frac{1}{2} \frac{(N+1)\alpha}{v} + \pi + \frac{\delta \alpha}{v} - \frac{.8 \text{Ifactor} \pi}{v} - \frac{\text{Ifactor} \delta \alpha}{v}
\]

The one variable missing a specific value for the process in treatment is \( N \), the sampling interval. As mentioned before, Taguchi finds the value for this parameter in his function by several approximations but the same result is found by minimizing the function using simple derivation. Thus, this is the method used to find the optimal sampling value \( N \) for the hole-boring process treated in this paper.

Through the use of Maple, the loss function for the off-line process as a function of \( N \) is:

\[
\lambda = 1680.0 \frac{1}{N} + 1.702031431 N + 9.514882005
\]

and its derivative with respect to \( N \), the interval size number is:

\[
d\lambda / dN = -1680.0 \frac{1}{N^2} + 1.702031431
\]

setting the derivative equal to zero to find a minimum value and solving for \( N \):

\[
31.41744424, -31.41744424
\]

so the optimal value for \( N \) is 31 (units).

Evaluating \( \lambda \) with \( N = 31 \), the result is:

\[
\lambda = 116.4714048
\]

116.47 yen/unit produced

so the hypothesized off-line quality control system would cost about 116 yen per unit produced. This is, indeed, about 90% more expensive than applying the on-line quality control method proposed by Taguchi.

One should remember that this hypothesized system included the use of only one X-bar chart. However, if it is combined with the use of an R-chart, a boon arises from the system. In using the two charts at the same time, one can monitor the mean and the vari-
 ance of the process. This is helpful because this could let the machine’s operators know that something may be going wrong, which would enable them to predict the occurrence of a problem and to try to prevent it. For example, an operator may see that several consecutive X-bar values are appearing above the target value and are going farther and farther away from it. At that point, he could decide to check the R-chart for discrepancies in the variability of the process. In case both are looking off-target, he may decide to check any operations or machine parts that are potential candidates for causing the problem. This would, in turn, allow the problem to be fixed before defective units are produced, which would save some quality control expenditures.

This quantity of savings is rather difficult to estimate without further information about the process. However, to get a feeling for how this may affect the quality control loss per unit produced, it may be assumed that this added advantage will prevent all costs (and savings) from being incurred (100/η)% of the times they are incurred, where η < 1. In this case one may consider a modified off-line quality control function as:

\[ \lambda = \frac{(2 + 0.8 m) \beta}{N} + \frac{1}{2} \left( \frac{(N+1) \alpha}{v} + \frac{\pi}{v} + \frac{\delta \alpha}{v} \right) \]

and

\[ \lambda = \frac{8 \text{factor} \pi}{v} - \frac{\text{factor} \delta \alpha}{v} (1 - \eta) \]

Considering different values for η, it is found that only η = 0.45 will make the on-line and the off-line loss functions about equal. This means that the use of the two control charts would have to let users predict and successfully correct any problems (without stopping the process) 45% of the average actual problems in the production line, a relatively high fraction.

IV. CONCLUSIONS

After the comparison of Taguchi’s Loss Function for an on-line quality control system to the adapted loss function for a hypothesized off-line quality control system, which consisted of an X-bar chart, it was proven that Taguchi’s argument was true for the studied process of hole boring at 1 Motor Company. The quality control cost found for the hypothesized process was nearly double the cost of Taguchi’s on-line system. In the consideration of the joint use of an R-chart along with the X-bar chart, it was estimated that a reasonably high efficiency in the problem-prediction facilitation of the modified off-line system would be required for the cost to be comparable to that of Taguchi’s off-line system.

It is important to make note of the reliability of the results found in this paper. Many assumptions were made to make a comparison of the off-line and on-line quality control systems feasible, the main one being that, under off-line control, the failures will be detected at the same time. Since Taguchi assumed that all units are defective after a defective unit is produced, the delay in the detection using an off-line control system will not be very significant. However, it is worth noting that Taguchi’s assumption will not be true for any process mainly due to its natural noise.

Due to the high degree of specificity of each process in which quality control methods are to be used, it is recommended to test off-line and on-line quality control methods before deciding which one to use. In doing this, further off-line quality control systems such as the use of Exponentially Weighted Moving Averages or Cumulative Sum (CUSUM) charts should also be considered since these have been proven to be faster in giving an “out-of-control” signal after something goes wrong with a process.

REFERENCES

ENDNOTES


iii In this context, the word attribute refers to any kind of labeling of a product, i.e. a satisfactory or unsatisfactory product.


viii Note that here $\sigma$ refers to the distribution of the pooled data in the control group and not to the distribution of the means $\sigma\sqrt{n}$


xi Taguchi finds the optimum measuring interval via several approximations. However, the same result is found by taking the derivative of the Loss Function and setting it equal to zero. See S. H. Park, G. G. Vining, Statistical Process Monitoring and Optimization, Marcel Dekker, New York, 2000, pp. 11 for Taguchi's approximation.


xiii He may probably mention it in the original paper on which the article is based, however, access to this original paper was not acquired.

Enhancing DNA Detection: Small-Molecule DNA Hybrid Model Studies

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The synthesis and characterization of a series of model compounds designed to simulate the core structure of small-molecule DNA hybrids (SMDHs) is described. Two different model compounds were synthesized using a solid-phase coupling technique: either benzyl alcohol or diphenylacetylene alcohol was first coupled to phosphoramidite and then to tetrahydrofuranyl alcohol before being oxidized to the corresponding benzyl phosphates. Both compounds were then subjected to a series of timed simulations designed to mimic the ammonium hydroxide deprotection step involved in the coupling of DNA to a small molecule and the subsequent exposure to UV irradiation that occurs during purification by HPLC or SDS-PAGE. With this strategy, we were able to demonstrate their robustness in the presence of concentrated ammonium hydroxide and under strong UV irradiation. Significantly, their stability as monomers underscores the overall stability of polymer-DNA hybrid materials that have been applied as a highly sensitive method for electrochemical DNA detection.

I. INTRODUCTION

Recent advancements in biomedical genetic research have led to the identification of specific genes which underlie complex and late-onset diseases. Early diagnosis and detection of debilitating diseases such as Parkinson’s, Alzheimer’s, and Huntington’s, offers patients the option of early treatment, and enables them to live longer and healthier lives. A crucial component of such preventive treatment, however, involves the reliable detection of disease-causing genes.

Many genetic diseases are often the result of faulty gene sequences caused by mutations. Accordingly, the detection of single-nucleotide polymorphisms (SNPs) in sequence-specific DNA strands has been crucial in the development of highly sensitive DNA assays. Current detection systems probe the presence of target DNA strands using a wide range of signaling mechanisms that include radioactivity, fluorescence, colorimetry, and electrochemistry to indicate the presence of target strands. Among these, electrochemical assays offer distinct advantages over other strategies due to their rapid detection, high sensitivity, and inherent ability to be engineered into affordable chip-based DNA biosensors.

Recently, the Nguyen group reported a novel electrochemical method for DNA detection through the use of polymer-DNA hybrid materials (Figure 1). They established that the specificity of DNA detection is greatly enhanced when multiple DNA strands are

Figure 1. A schematic drawing of a polymer-DNA hybrid. The vertical line represents the organic polymer chain. The helical lines on the side represent single DNA strands.
linked as side chains of a polymer. In particular, these polymer-DNA hybrids exhibit higher dehybridization temperatures and unusually sharp melting profiles compared to simple DNA duplexes (Figure 2). Such enhanced thermal properties are highly desirable because probe materials with increased dehybridization temperatures and sharp melting properties have shown to be very selective in the detection of SNPs in disease-causing genes.8.9

However, while it has been hypothesized that the enhanced recognition properties of the Nguyen polymer-DNA hybrids are due to the presence of multiple DNA linkages in close proximity, the parameters which govern the enhanced melting behavior such as the number of DNA strands per polymer, distance between adjacent DNA strands, and the geometrical placement between strands remain unknown quantities. To understand the relationship between the structure of the polymer-DNA hybrids and their observed enhanced melting transition, we designed and synthesized a series of small-molecule DNA hybrids (SMDHs) (Figure 3).10

A core phenylacetylene unit was specifically chosen as the molecular scaffold in order to provide a rigid linker between the DNA strands and the molecular core. This allowed the precise control over the geometrical place-

Figure 2. Panels 2A and 2B show the thermal denaturation curves of polymer-DNA:polymer-DNA aggregates and panel 2C shows the thermal denaturation curve for duplex DNA formed from plain oligonucleotides with the same base sequence. Compared to panel 2C, the polymer-DNA:polymer-DNA aggregates show a much higher melting temperature and a sharper melting transition.

Figure 3. Examples of small-molecule-DNA hybrids (SMDHs).
Figure 4. Panel 4A shows the gel results obtained when the SMDH reaction mixture was purified using SDS-PAGE. The band corresponding to the SMDH, with 3 DNA strands was cut out and loaded into Lane 1 of a second gel. Panel 4B shows the results of the second gel and the products of the SMDH degradation. As shown in Lane 1, the SMDH degraded into the SMDH with only 2 DNA strands and SMDH with only 1 DNA strand attached.

Studying the thermal behavior of well-defined structures like SMDHs offers two significant advantages over the ill-defined polymer-DNA hybrids. First, by analyzing the melting profiles of aggregates made from SMDHs, we can quantify and optimize the parameters which influence the cooperativity observed in the polymer-DNA hybrid system. Understanding these underlying parameters will allow us to develop a model that can be applied to optimize the design of small molecular probes involved in the specific targeting of DNA and proteins. Second, if the SMDHs exhibit enhanced melting, we can apply this technology to the development of a new class of organic-hybrid materials that can be easily engineered into chip-based DNA biosensors.

Interestingly, during the purification and characterization of the SMDHs by standard High Performance Liquid Chromatography (HPLC) and Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) techniques, we observed that the SMDH molecule appeared to lose DNA strands from the molecular core (Figure 4). Given the nature of the DNA synthesis procedure and the subsequent purification processes, we hypothesized that the observed degradation was either due to: (1) cleavage of the benzyl ether linkage during the ammonium hydroxide deprotection step in DNA synthesis (Figure 5) or (2) degradation caused by UV photolysis of the phenylacetylene linker during purification by HPLC or SDS-PAGE (Figure 6).

Herein, we describe a series of model studies to investigate the stability of the SMDH system under relevant conditions. We demonstrate the stability of two model compounds in the presence of ammonium hydroxide and under UV irradiation through timed simulated conditions as found in standard DNA synthesis and purification techniques. More importantly, because the rigid diphenylacetylene unit in our model compound H is present as a side chain of the monomeric unit in the polymer-DNA hybrid (Figure 7), the robustness of the model compounds has significant implications on the overall stability of the polymer-DNA hybrid system.

II. MATERIALS AND METHODS

Synthesis of Model Compound C
The simplest model compound, C, was synthesized through a modified literature procedure for solid-phase phosphoramidite coupling.11 The benzyl alcohol was first reacted with an excess of cyanoethyl N,N-diisopropyl phosphoamidochloridite until all the alcohol had been consumed. The resulting phosphoramidite intermediate (A) was then coupled with tetrahydrofurfuryl alcohol to yield the desired phosphotriester (B)
Figure 5. Hypothesized mechanism for the base-catalyzed cleavage of the benzyl ether and furanyl ether linkages.

Figure 6. Proposed mechanism for the radical cleavage of the benzyl ether bond.

Figure 7. A schematic drawing of the precursor to the Nguyen polymer-DNA hybrid prior to DNA coupling. The side chain contains the rigid diphenylacetylene linker found in model compound H.
Scheme 1.

A. Coupling

![Chemical structure](Scheme1A)[1]

B. Oxidation

![Chemical structure](Scheme1B)[3]

(Scheme 1A), which was oxidized to C using sodium periodate ions immobilized on an Amberlyst A-26 resin (Scheme 1B). 1H NMR (400 MHz, D2O): δ 1.87 (m, 2H, furan CH3), 1.98 (m, 2H, furan CH3), 2.84 (dd, 2H, cyanomethyl CH2, J = 5.2 Hz, J = 6.4 Hz), 3.78 (m, 2H, furan CH3), 3.96 (m, 1H, furan CH), 4.11 (dd, 2H, furan linkage CH2, J = 5.6 Hz), 4.24 (dd, 2H, cyanomethyl CH2, J = 6.8 Hz, J = 12.8 Hz), 5.19 (d, 2H, benzyl CH2, J = 10.4 Hz), 7.47 (m, 5H, Ar-H). 31P NMR (400 MHz, D2O): δ −1.02. ESI-MS: Exact mass calc'd for [C15H12NO5P]: 425.11. Found: 425.2.

Synthesis of Model Compound H

The diphenylacetylene alcohol E required for the synthesis of H was obtained from the base-catalyzed hydrolysis of D, which was obtained from the Sonagashira coupling of phenylacetylene with 4-iodobenzyl acetate (Scheme 2A). The phosphoramidite intermediate F was formed through the coupling of E to cyanomethyl N,N-diisopropyl phosphoamidochloridite. F was then coupled to tetrahydrofurfuryl alcohol to form G which was subsequently oxidized to yield the desired phosphate H using a similar procedure as described in the synthesis of C (Scheme 2B). 1H NMR (400 MHz, CDCl3): δ 1.64 (m, 2H, furan CH3), 1.95 (m, 2H, furan CH3), 2.74 (dd, 2H, cyanomethyl CH2, J = 2.8 Hz, J = 6.0 Hz), 3.80 (m, 2H, furan CH3), 3.86 (m, 1H, furan CH), 4.08 (m, 2H, furan linkage CH2), 4.22 (m, 2H, cyanomethyl CH2), 5.14 (dd, 2H, benzyl CH2, J = 5.6 Hz, J = 8.0 Hz), 7.36 (m, 5H, Ar-H), 7.54 (m, 4H, Ar-H). 31P NMR (400 MHz, CDCl3): δ −0.914. ESI-MS: Exact mass calc'd for [C22H23NO5P]: 425.14. Found: 425.78.

Degradation Studies of Model compounds C and H during Basic Cleavage

To test the stability of C and H under conditions which mimic the basic cleavage deprotection step that occurs during standard DNA coupling (Scheme 3), we exposed each model compound to 1M ammonium deuterioxide in D2O at room temperature for 2 h to yield the deprotected compounds X and Y, respectively. The extent of deprotection was monitored by taking 1H NMR spectra of the reaction mixture at regular time intervals of 15 min. After 2 h, the ammonium deuterioxide was removed under vacuum, and the resulting solids were characterized using ESI-MS as well as 1H and 31P NMR spectroscopy.

Reaction mixture of Compound C after deprotection (after 2 h)

1H NMR (400 MHz, D2O): δ 1.98 (m, 2H, furan CH3), 2.25 (m, 2H, furan CH3), 4.08 (m, 1H, furan CH),
Scheme 2.

A. Synthesis of Starting Monomer E

\[
\text{Ph} + \text{PhCO} \xrightarrow{1\% \text{PdCl}_2(\text{PPh}_3)_2, 2\% \text{Cu}, \text{TEA}} \xrightarrow{\text{NaOMe, MeOH}} \text{PhOH}
\]

B. Coupling

\[
\text{PhOH} + \text{N} = \text{CH-CO-P-N} \xrightarrow{\text{CH}_2\text{Cl}_2, \text{Hünig's Base}} \xrightarrow{\text{HO-} \xrightarrow{\text{CH}_2\text{Cl}_2}} \text{Ph}
\]

C. Oxidation

\[
\text{Ph} \xrightarrow{\text{IO}_4^- \text{(Amberlyst A-26)}} \text{Ph}
\]
Scheme 3.

Basic Deprotection of Model Compound C

Basic Deprotection of Model Compound H

4.16 (m, 2H, furan CH₂) 4.41 (d, 2H, furan linkage CH₂), 5.29 (d, 2H, benzyl CH₂ J = 7.2 Hz), 7.82 (m, 5H, aromatic H). 31P NMR (400 MHz, D₂O): δ 1.08. ESI-MS: Exact mass calc'd for [C₇H₄O₄P]: 272.08. Found: 272.33.

Reagents mixture of Compound H after deprotection (after 2 h)
1H NMR (400 MHz, CDCl₃): δ 1.48 (m, 2H, furan CH₂), 1.85 (m, 2H, furan CH₂), 3.72 (m, 1H, furan CH), 3.78 (m, 1H, furan CH), 3.99 (d, 2H, furan linkage CH₂, J = 7.2 Hz), 4.92 (d, 2H, benzyl CH₂ J = 6.0 Hz), 7.34 (m, 5H, Ar-H), 7.50 (m, 4H, Ar-H). 31P NMR (400 MHz, CDCl₃): δ 0.297. ESI-MS: Exact mass calc'd for [C₇H₄O₄P]: 372.11. Found: 372.73.

Stability of each model compound in the presence of ammonium hydroxide was also determined by leaving the deprotection mixture in aqueous ammonium hydroxide for an extended period (1 week for C and 2 days for H). Subsequent comparative analysis of the reaction mixture by ESI-MS as well as 1H NMR and 31P NMR spectroscopy verified the stability of X and Y.

UV Photolysis Studies of Model compounds X and Y
The effects of UV irradiation on compounds X and Y were assessed by exposing their solutions (X in deuterium oxide and Y in deuterated methylene chloride) in quartz NMR tubes to UV light from a Xenon lamp (Ushio UXL-151HO) for specific time periods of 5 min, 15 min, and 20 min to simulate conditions that occur during the SDS-PAGE and HPLC purification process.

Model compound X' (after 20 min of UV exposure)
1H NMR (400 MHz, D₂O): δ 1.57 (m, 2H, furan CH₂), 1.89 (m, 2H, furan CH₂), 3.71 (m, 2H, furan CH₂), 3.80 (m, 1H, furan CH), 3.84 (d, 2H, furan CH₆, J = 6.8 Hz),
Model compound Y' (after 20 min of UV exposure)

{1H NMR (400 MHz, CD3Cl): δ 1.60 (m, 2H, furan CH2), 1.89 (m, 2H, furan CH2), 3.79 (m, 2H, furan CH2), 3.91 (m, 1H, furan CH), 4.07 (t, 2H, furan linkage CH2), 5.04 (d, 2H, benzyl CH2, J = 7.2 Hz), 7.37 (m, 5H, Ar-H), 7.52 (m, 4H, Ar-H). {31P NMR (400 MHz, CD3Cl): δ 0.297. ESI-MS: Exact mass calcd. for \( [C_{5}H_{9}O]^{+} \): 272.08. Found: 272.35.

After each time period, a {1H NMR spectrum was taken to monitor if degradation of the compound had occurred. ESI-MS and {31P NMR spectra were also obtained along with {1H NMR spectra upon completion of the experiment. To prevent thermal degradation during each period of UV irradiation, the temperature of each NMR tube was maintained through the use of a continuously blowing fan in the experimental set-up. Each NMR tube was also wrapped tightly in aluminum foil to keep the compound away from external UV light sources when the experiment was not being conducted.

III. RESULTS AND DISCUSSION

Syntheses of Model Compounds C and H

To analyze the stability of the SMDHs, compounds C and H were designed to model the simplest two unit structures of the SMDHs: the benzyllic and the diphenylacetylene benzyllic linkages. Model compound C contained only the benzyl ether linkage attached to the phosphoramidite and was used to determine the effects of concentrated ammonium hydroxide on the stability of the ether bond. Model compound H was designed to probe the effects of UV irradiation on the stability of the diphenylacetylene benzyl ether linkage. The successful synthesis of both compounds was established through ESI-MS as well as {1H and {31P NMR spectroscopy (Table 1).

Basic Cleavage Degradation Study

The synthesis of SMDHs in the Nguyen group is currently based on standard solid-phase oligonucleotide techniques. The first protected nucleoside is pre-attached to a controlled-porosity glass resin bead and is then coupled to the small molecular core to serve as the initial building block for DNA chain extension. Upon completion of DNA synthesis, the linker between the oligonucleotide and the solid support is cleaved through addition of concentrated aqueous ammonia. The ammonium hydroxide also serves to deprotect the phosphate backbone through removal of the cyanoethyl group. Accordingly, we hypothesized that the presence of concentrated aqueous ammonium hydroxide could have contributed to the cleavage of the benzyl ether bond (Figure 5).

As expected, our results (Table 1) showed that the reaction of both model compounds C and H with ammonium hydroxide successfully removed the cyanoethyl groups (Figure 8). Specifically, the {1H NMR

<table>
<thead>
<tr>
<th>Compound</th>
<th>{1H-NMR Chemical Shift (benzyl ether proton)</th>
<th>Mass Obtained</th>
<th>Theoretical Mass</th>
</tr>
</thead>
<tbody>
<tr>
<td>Model compound C</td>
<td>δ 5.19 (doublet, J = 10.4 Hz)</td>
<td>325.20</td>
<td>325.11</td>
</tr>
<tr>
<td>After deprotection (2h)</td>
<td>δ 5.29 (doublet, J = 7.2 Hz)</td>
<td>272.33</td>
<td>272.08</td>
</tr>
<tr>
<td>Pure benzyl alcohol</td>
<td>δ 4.64 (singlet)</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Model compound H</td>
<td>δ 5.14 (doublet of doublets,</td>
<td>425.78</td>
<td>425.14</td>
</tr>
<tr>
<td></td>
<td>J1 = 5.6 Hz, J2 = 8.0 Hz)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>After deprotection (2h)</td>
<td>δ 4.92 (doublet, J = 7.2 Hz)</td>
<td>372.73</td>
<td>372.11</td>
</tr>
<tr>
<td>Pure diphenylacetylene alcohol</td>
<td>δ 4.61 (singlet)</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>
decrease in height of the cyanoethyl proton peaks and a corresponding growth of new peaks further upfield as the reaction progressed with time. We also observed that the benzyl ether proton peak only shifted slightly (δ 5.19 for C to δ 4.92 for X; δ 5.06 for H to δ 4.92 for Y) after the deprotection step. Furthermore, while the benzyl ether peak in compound H was observed to be two overlapping doublets (due to diastereotopic protons), the benzyl ether peak in Y was observed to be a doublet.

Our results indicated that the addition of ammonium hydroxide during the coupling and deprotection step did not cause cleavage of the benzyl ether bond in either model compound. If the benzyl groups had cleaved off during the reaction, we would have expected significant shifts in the resonances for the benzylic protons (δ 5.19 for C to δ 4.64 for benzyl alcohol, δ 5.06 for H to δ 4.61 for diphenylacetylene benzyl alcohol) and a concurrent loss of phosphorous coupling (i.e., conversion from a doublet to a singlet). Instead, the slight shifts in the phosphorous-coupled benzyl ether proton peaks observed can be attributed to a change in the chemical environment of the benzyl ether protons after the removal of the cyanoethyl group.

The stability of the model compounds in the presence of ammonium hydroxide was also verified by ESI-MS which yielded the expected masses of compounds X and Y. Hence, the stability of our model compounds in ammonium hydroxide indicated that the deprotection step involved in the coupling of DNA chains to the small molecular core was unlikely to have caused the observed loss of DNA strands.

**UV Irradiation Photolysis Study**

During the synthesis of SMDHs, the desired product is usually purified through the use of standard HPLC or SDS-PAGE techniques after coupling of the small molecules to DNA. In particular, the purification of the SMDH by HPLC utilizes a UV-absorbance detector to monitor the presence of the product peaks. While the irradiation of the sample with UV light during HPLC only occurs over a few seconds, the dilution of the samples allows almost every molecule to be irradiated or activated.

Likewise, during the purification of the SMDH using SDS-PAGE, exposure to UV irradiation is necessary to allow the determination of both the location and size of the purified product on the gel slab. To this extent, the presence of UV-active phenylacetylene linkers in the SMDHs could have potentially contributed to the activation and cleavage of the benzyl ether bond when exposed to UV light to yield a stable benzylic radical (Figure 6).

However, the results of our photolytic degradation study showed that the benzyl ether linkage in both compounds X and Y was stable under UV irradiation. Within experimental error, the benzyl ether proton peaks in the 'H NMR spectra of both model compounds X and Y remained as phosphorous-coupled doublets whose positions did not change significantly even after 20 min of UV irradiation (Table 2).

More importantly, degradation of the model compounds would have resulted in the free alcohols. In the presence of a high concentration of water molecules, the abstraction of an H atom from the solvent by the benzyl radical would have been expected to occur at a much faster rate than the combination of two radicals,
Table 2. Summary of results obtained for the UV irradiation photolysis study.

<table>
<thead>
<tr>
<th>Compound</th>
<th>1H-NMR Chemical Shift (benzyl ether proton)</th>
<th>Mass Obtained</th>
<th>Theoretical Mass</th>
</tr>
</thead>
<tbody>
<tr>
<td>Model compound X</td>
<td>δ 5.29 (doublet, J = 7.2 Hz)</td>
<td>272.33</td>
<td>272.08</td>
</tr>
<tr>
<td>Compound X' (after 20 min UV irradiation)</td>
<td>δ 4.92 (doublet, J = 7.2 Hz)</td>
<td>272.35</td>
<td>272.08</td>
</tr>
<tr>
<td>Pure benzyl alcohol</td>
<td>δ 4.64 (singlet)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Model compound Y</td>
<td>δ 4.92 (doublet, J = 7.2 Hz)</td>
<td>372.73</td>
<td>372.11</td>
</tr>
<tr>
<td>Compound Y' (after 20 min UV irradiation)</td>
<td>δ 5.04 (doublet, J = 7.2 Hz)</td>
<td>372.31</td>
<td>372.11</td>
</tr>
<tr>
<td>Pure diphenylacetylene alcohol</td>
<td>δ 4.61 (singlet)</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

driving the equilibrium reaction towards formation of the free alcohol.

Thus, by comparing the ESI-MS data before and after UV irradiation and noting the absence of any degraded phosphate fragments, we concluded that UV irradiation had no significant effect on cleavage of the benzyl ether bond. In fact, because our model compounds were stable even after UV irradiation for longer periods of time than experienced during SMDH purification (few seconds vs. 20 minutes), our results suggest that short exposures to UV light in the HPLC and SDS-PAGE purification processes are unlikely to cause cleavage of the benzyl ether linkage.

IV. CONCLUSION

Our model studies demonstrate the stability of the benzyl and diphenylacetylene unit structures of the SMDH in the presence of concentrated ammonium hydroxide and under UV irradiation. In particular, because compound H is iso-structural with the side chains of the Nguyen polymer-DNA hybrids (i.e., the polymer-DNA hybrids utilize the rigid diphenylacetylene as a monomeric side chain to attach DNA strands to the polymeric backbone), our results imply an overall stability for this class of materials. Significantly, it suggests that the DNA chains coupled to the Nguyen polymer-DNA hybrids are unlikely to be cleaved during purification and characterization.

We note however, that our two model compounds only contain a simple 5' furanyl phosphate ester substituent in each, in contrast to two (one 3'-substituted and the other 5'-substituted) in DNA single strands. The presence of a complex length of DNA attached to the small molecular core in the SMDH, could also have contributed to further destabilization effects that would have led to the cleavage of the furanyl substituents from the phosphate backbone under relevant conditions. Furthermore, the attachment of two more phenylacetylene groups on SMDH, could also have contributed to a more delocalized core electronic structure and an easier activation of the benzyl ether bond than the simplest case modeled in compound Y. Hence, further work to elucidate the observed degradation in the SMDH, molecule will include the design of a trimer model compound that is coupled to a single furanyl phosphate ester and a trimer model compound coupled to three furanyl phosphate ester units.

Acknowledgements

D.K. acknowledges the Northwestern McCormick Corporate Partner Research Grant, the American Foundation for Aging Research, the McCormick Sara Boley Fund, and the Sigma Xi Scientific Research Society for financial support of her research. She also thanks Professor SonBinh T. Nguyen, Professor Suzanne A. Olds, Mr. Brian R. Stepp and Dr. Julianne M. Gibbs-Davis for helpful discussions and Ms. So-Hye Cho for the acquisition of ESI-MS data.
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High-Throughput Protein Degradation Assays

Protein degradation is an integral step in several cellular processes, including cell cycle control, gene expression, antigen presentation, and signal transduction. Aberrant degradation is associated with such diseases as Alzheimer’s disease, prion disease, and Huntington’s disease. We are focusing on characterizing selectivity in protein unfolding and destruction by ATP-dependent proteases to determine whether proteases differ in their ability to degrade proteins. We worked to adapt a biochemical protein degradation assay used to measure protease unfolding strength to high throughput automation. The results indicate that the developed methodology decreases experimental error that accrues by pipetting at individual timepoints during the biochemical assay, increases the general ease of experimental operation, and allows an increased throughput, thereby facilitating large scale proteomic studies. Additional high-throughput degradation assay techniques will be developed for future experiments involving the characterization of ATP-dependent proteases.

I. INTRODUCTION & BACKGROUND

In order to be active, proteins must fold into well-defined three-dimensional structures. However, the unfolding and deactivation of proteins are also extremely important, if not central to, cellular processes such as gene expression, cell cycle control, signal transduction, development, and antigen presentation. Faulty unfolding and degradation is associated with diseases as Alzheimer’s disease, prion disease, and Huntington’s disease.

The unfolding and degradation of proteins is a multistep process. Proteases first bind the protein destined for degradation on the protease chaperone ring where the protein is recognized by its targeting signal. The chaperone ring then unfolds the protein sequentially along its polypeptide chain (from the protein’s point of attachment to the protease to the opposite terminus) in a process that requires ATP hydrolysis. The unfolded polypeptide is translocated one amino acid at a time to a central proteolytic chamber where it contacts the proteolytic sites and is degraded rapidly, releasing short peptide fragments.

In order for the cell to degrade the appropriate protein at the right time and to not disturb the activities of other proteins in the cell, proteases need to be highly selective in what substrates they degrade. Selectivity for degradation is achieved in part by the overall architecture of the protease. ATP-dependent proteases are composed of two functionally distinct domains: the chaperone domain and the proteolytic domain. The chaperone rings sit on either side of the proteolytic chamber and regulate degradation. The chaperone rings that cap the central proteolytic domain bind the protein targeted for degradation and unfold it in preparation for the proteolytic step. The proteolytic rings house the active sites of proteolysis. The proteolytic chamber allows regulation and selectively of protein degradation in two ways. First, the chamber sequesters the active sites of proteolysis from the cytoplasm. Second, the proteolytic chamber bestows selectivity to the degradation process by restricting the entry of folded proteins. The entryway to the proteolytic chamber is typically 10-20 Å, a diameter too narrow to allow the passage of even the smallest of globular proteins. Thus, the chamber’s structure sterically restricts entry of folded proteins and therefore imposes an unfolding requirement before the degradation of proteins can occur. This regulation allows the ability to unfold a protein to differ among proteases.

However, selectivity in proteolysis is not derived from the architecture alone; there is also a level of selectivity on the part of the binding of the substrate.
ATP-dependent proteases recognize proteins to be unfolded by targeting signals that vary in length and are often specific to the unfoldase. Many ATP-dependent proteases, including ClpAP and ClpXP, exhibit selectivity by an affinity for targeting signals located at the protein’s N-terminus,7 and C-terminus.8 Targeting signals do not always have a strong affinity for the protease. In fact, numerous short (i.e., three amino acids) and variable peptide sequences have been shown to target proteases such as ClpXP. In addition, the same targeting signal can target several different proteases.9 Further, it has been shown that one can fuse a targeting signal that destines a protein for a different cellular compartment onto a carrier protein and convert that protein into a degradation precursor. For example, the Matouschek Laboratory fused the first 65 amino acids of cytochrome b₃, a protein that is normally destined for the mitochondria, to the ribonuclease barnase, and demonstrated proteolysis by the ClpAP and HslUV proteases in vitro, suggesting that targeting sequences for proteases are not always highly specific.10

We proposed that an additional layer of selectivity in proteolysis may result from the unfolding strength of proteases. For instance, a mechanism may exist where the cell can upregulate the concentration of a weak protease to selectively eliminate unstable/misfolded proteins and, at the same time, avoid eliminating stable proteins that may have the same targeting signal (because the protease is too weak to unfold them). This model relies on ATP-dependent proteases actually having substantially different unfolding abilities. We, therefore, set out to measure the unfolding abilities of all known proteases. For this large scale analysis, we wished to utilize high throughput analysis. The project described below explains how we developed this technique of high throughput analysis.

In this study, a multi-channel pipetting technique was first developed to facilitate adapting the manual assay’s protocol to robotics. The eventual implementation of robotics has several advantages: the robotic setup (1) decreases experimental error that accrues during the pipetting of individual timepoints during the biochemical assay, (2) increases the general ease of experimental operation, and (3) allows an increased throughput, thereby facilitating large scale proteomic studies. We compared protein degradation assays with model protein assays completed by the manual individual pipette technique, the multi-channel pipette technique, and the robotic multi-channel pipette technique and successfully applied the degradation assay setup to robotics. In the future, we will implement additional methods of automation to improve other aspects of this assay.

II. MATERIALS & METHODS

In order to measure the unfolding abilities of all known proteases, biochemical degradation assays were performed under several varying conditions such as temperature, stability of the substrate, and enzyme concentration for each of the known ATP-dependent proteases. Briefly, reactions were carried out by mixing in vitro radioactively labeled substrates to the purified enzyme and adding ATP (ATP is required to start the reaction, because these enzymes are ATP-dependent). At designated timepoints, a fraction of the reaction was withdrawn, and the reaction was quenched in stop buffer. After this, the reaction products were analyzed by SDS-polyacrylamide gel electrophoresis and autoradiography. Because we wanted to perform this assay under many different conditions, we knew high-throughput analysis would be extremely beneficial. The process of developing this method of high-throughput analysis is explained below.

III. SPECIFICS OF THE DEGRADATION ASSAY

Creation of substrates for degradation

A model multi-domain protein substrate (which included a targeting signal) was prepared to characterize the unfolding ability of the HslUV protease. Many other substrates were used in various experiments that target each of the other proteases from either their N-termini and C-termini. The substrate for HslUV was constructed from E. coli dihydrofolate reductase (eDHFR), a stable protein domain,11 and barnase, an easy-to-unfold ribonuclease from Bacillus amylo-lactucae, which were connected via a six amino acid poly-glycine linker.12 In order to target these proteins C-terminally for degradation by HslUV, SulA, a natural substrate for HslUV, was tethered to the construct at its C-terminus by a nine amino acid GGSGGTTGGS linker.13 The final substrate construct consisted of the targeting signal SulA, followed proximally by barnase and distally by DHFR (N—DHFR—Barnase—SulA—C).
The gene encoding this model protein was cloned into the plasmid vector pGEM (Promega) for \textit{in vitro} protein production and verified by DNA sequencing. The DNA construct was linearized to increase transcription efficiency, and the linearized DNA was transcribed by \textit{in vitro} transcription (Ribomax, Promega). Next, the mRNA was translated in a rabbit reticulocyte extract (Flexi Rabbit Reticulocyte Lysate, Promega), a source of the translation machinery such as ribosomes and amino acids, in the presence of $[^3S]$ methionine. Labeling substrates allowed analysis of substrate degradation by SDS-PAGE and electronic autoradiography (Instant Imager, Packard). Finally, substrate proteins were partially purified by removing partially translated products associated with ribosomes via high-speed ultracentrifugation and ammonium sulfate precipitation to remove partially translated products.\textsuperscript{16} The result was a 50-250 nM substrate protein that was detectable by autoradiography (Figure 1).

\textbf{General Degradation Assay Conditions}

The proteolytic degradation assay was performed in HsUV degradation buffer (25 mM Tris-HCl [pH 7.8], 150 mM KCl, 1 mM DTT, 5 mM MgCl$_2$), an ATP regenerating system (20 mM creatine phosphate, 0.1 mg/mL creatine kinase, 4 mM ATP). We used 0.05 \mu M HsUV enzyme. 50 nM \textit{in vitro} substrate translation reaction was precipitated by centrifugation at 18,000 x g for 15 minutes at 4°C.

\textbf{Manual Assay}

The manual degradation assay was done with one pipette by hand and was the first methodology developed in this laboratory to complete this type of assay. In the case of one reaction, the manual individual pipette degradation assay was initiated by prewarming the reaction mixture on an aluminum heat block in a 30°C circulating water bath. Eight eppendorf tubes were set up to with 10 \mu L of loading (stop) buffer in each tube (Figure 2a). 10 \mu L of substrate were added the reaction tube.

When the next timepoint was ten seconds away for the first reaction, 4 \mu L of reaction mixture were withdrawn, and the reaction was quenched in stop buffer at the time point. This quenching process was repeated at designated timepoints (0', 5', 10', 15', 30', 60', 120', 150') for each reaction. When the time course was complete, the reaction mixtures were loaded into a SDS-Page Gel, and the reaction’s successive timepoints were loaded individually with increasing timepoints loaded from left to right. When completing more than one reaction, the reactions were completed using staggered timepoints because there was no way for an experimenter to complete simultaneous reactions by hand. With staggered timepoints, the experimenter had enough time to prepare for each reaction’s timepoint.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{preparation.png}
\caption{Clone, Transcribe, and Translate. An SDS-page gel was completed to test the purity of the translation. A \textsuperscript{15}S-50 nM substrate protein was detectable (bottom).}
\end{figure}
Figure 2. Manual & Multichannel Assay Setup. The manual assay used eight eppendorf tubes while the multichannel assay used a 96-well PCR plate and multichannel pipettman to allow the immediate advantage of eliminating staggered timepoints and completing simultaneous reactions to increase throughput.

Figure 3. Multichannel Assay & Gel Loading. 4 μl of the reaction mixture are withdrawn (left) and quenched in stop buffer. After the assay is complete, the samples are loaded into a 9-well SDS-page gel with the multichannel pipette (right).
Multichannel Assay

Development of the manual multichannel pipette degradation assay allowed the immediate advantage of eliminating staggered timepoints and completing simultaneous reactions to increase throughput. The manual multichannel pipette degradation assay began with eight different reactions in an eight-tube strip pre-warming in an aluminum heat block in a 30°C circulating water bath. These strip-tubes were used because they allow one to handle numerous reactions simultaneously. Instead of setting up 24 eppendorf tubes with 10 μL of loading (stop) buffer in each tube, as in the manual assay, the eight rows of a 96-well full skirted PCR plate were filled with 10 μL of loading (stop) buffer (Figure 2b). This plate was easier to handle when completing the timesteps, could be placed in the thermocycler in order to warm the samples before they were loaded into a gel, and could be translated to robotics more easily. 10 μL of substrate were added to the reaction tubes with a 10 μL 8-channel pipette to start the reaction. The reaction was then mixed with a 50 μL 8-channel pipetteman set to 50 μL. This step avoided the spin down process and the use of caps; thus, the process could easily be converted to robotics.

When the timepoint was ten seconds away, 4 μL of reaction mixture were withdrawn with the 10 μL eight-channel pipetteman (Figure 3). The reaction was quenched in the vertical time point tube #1 of each strip with the pipetteman turned vertically so that each gel would show the results of one reaction at successive timepoints (Figure 2b). This quenching process was repeated for each timepoint. When the time course was complete, the 96-well PCR plate could be immediately loaded into a SDS-Page Gel with the eight-channel pipetteman (Figure 3).

Robotic Assay

The robotics degradation assay was completed in a similar fashion, a liquid handling robot (the Packard MultiPROBE II EX HT) completed the assay on its own so there was no need for the experimenter to complete a timepoint every half hour. With the high-throughput assay, the experimenter could set up the reaction and return in a few hours to find a completed degradation assay.

One 8-tube strip of low-profile strip tubes filled with 10 μL substrate in each tube was placed in column one of the 96-well aluminum heating block tower. One empty 8-tube strip was placed in column two of the 96-well aluminum heating block tower (Figure 4). These low-profile tubes had a lower clearance height than regular strip tubes to avoid a collision by the MultiPROBE II. The robot was also programmed to load the enzyme solution and the loading buffer for
the experimenter. Two 1.5 mL eppendorf tubes filled with one mL of loading (stop) buffer were placed in rows three and four of column two of a clear eppendorf tube rack on the robot deck. One 1.5 mL eppendorf tube filled with 500 μL of enzyme solution was placed in row four of column one of the rack. The robot’s eight-channel pipetteMan dispersed 10 μL of loading (stop) buffer in each well of the 96-well PCR plate. 20 μL of the enzyme mixture were pipetted and placed into an empty low-profile strip tubes in column two of the aluminum heating block. This solution was then allowed to pre-warm for three minutes. After the pre-warm step was complete, 50 μL of the enzyme solution was added to the substrate filled strip tubes in column one. This mixture was then mixed by the robot’s pipetteMan, and at the defined timepoint (in this case, timepoint 0), the robot withdrew 4 μL of substrate/reaction mixture and quenched the reaction in column one of the 96-well PCR plate filled with loading (stop) buffer. This quenching process was repeated for each timepoint. The same gel loading procedure as in the manual multichannel assay was then completed.

**IV. RESULTS**

By qualitative analysis, one can see that degradation and degraded product accumulation occurred in all three assays. The top band of each gel showed a decrease in detected radioactivity as time passes, illustrating the degradation of the full length fragment of substrate, while the bottom band showed an increase in detected radioactivity as time passes, illustrating the accumulation of cDHFR (~50 nM), a fairly stable peptide that is placed N-terminally in the model substrate. Some degradation of cDHFR did occur however, accounting for the decreased detection of radioactivity as the timepoints progress in the bottom band of the sample without methotrexate. After the addition of methotrexate, a ligand that further stabilizes cDHFR, the recovery of fluorescence in the bottom band of the sample with methotrexate increased. The nonlinear least square fittings of the Michaelis-Menten equation were also determined using the Kaleidagraph program (Synergy) for qualitative analysis. The same degradation kinetics are observed in all three methodologies (Figure 5).

**Figure 5.** Comparison of Manual, Multichannel, and Robotic Assays. A ligand (MTX) was used to stabilize the eDHFR domain so the formation of a distinct eDHFR product could be observed (bottom band of each gel). Autoradiography of SDS-page gels run with standard markers and the nonlinear least square fittings of the Michaelis-Menten equation (determined using the Synergy Kaleidagraph program) were used to analyze the degradation products. These data illustrate that the multichannel and robotic assays successfully translate to automation as the same degradation kinetics are observed in all three methodologies.
V. DISCUSSION & CONCLUSIONS

Protein unfolding by ATP-dependent proteases is an integral step in several cellular processes, including cell cycle control, signal transduction, and antigen presentation. However, without highly reproducibility of data collection and experimental set-up, characterization of these ATP-dependent proteases is tedious. The high-throughput assay decreases experimental error that accrues by pipetting of individual timepoints during the biochemical assay, increases the general ease of experimental operation, and allows an increased throughput, thereby facilitating large scale proteomic studies. The SDS-page gels of the multichannel and robotic assays mirrored the gels of the manual assay. These data illustrate that the multichannel and robotic assay successfully translates to automation as the same degradation kinetics are observed in both methodologies.

In addition, the multichannel and robotic assays also showed decreased experimental error caused by the time-constrained movements of the researcher. Completing timepoints with the manual assay was tedious because accuracy in quenching the reaction mixture at the exact second of the next timepoint was close to impossible with the time-constrained movements of the human hand. The multichannel assay helped decrease these time-constrained movements so that a timepoint for eight reactions could be completed all at the same time. In this way, the staggered timepoints that were a part of the manual assay were eliminated so that closer timepoints were made feasible. If the timepoint was completed slightly before or after the timepoint was supposed to be completed, all eight reactions had identical rates of error rather than each individual reaction having distinct amounts of error with the manual assay. The robotic assay completely eliminated the time-constrained movements of the human hand and replaced them with the extremely accurate programmed movements of the robotic probes, thus additionally increasing the timepoint accuracy and creating systematic error rather than random error as in the manual assay.

The data yield was also increased by the multichannel and robotic assays. Only three reactions at eight timepoints could be tested comfortably with the manual degradation assay as a result of the time-constrained movements of the human hand and the individual pipetting required with this assay. On the other hand, eight reactions as eight timepoints were tested with the multichannel and robotic assays, exhibiting a clear increase in data yield. After these experiments were completed, additional PCR plate decks and reaction plate decks were added onto the MultiPROBE operation deck in order to further increase the data yield produced by the robotic technique.

In the future, we hope to automate SDS-PAGE gel loading with robotic gel loading. In this way, the entire degradation assay process will be faster and more accurate. The SDS-PAGE gel step and any error associated with the completion of timepoints may eventually be eliminated by developing real-time fluorescence degradation assays that are readable by a 384-well TECAN GENios Pro Plate Reader. Using GFP fluorescence detection within degradation assays would eliminate the use of radioactive labeling, allowing a safer alternative to protein visualization. Attempts in expanding this robotic experimental set-up involving eukaryotic proteasome assays have been successful thus far. We also hope to increase the robotic assay data yield further by adding additional PCR plate decks and reaction plate decks.

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The author, George Desh, is a third-year biomedical engineering student in the McCormick School of Engineering at Northwestern University. His primary research interest is in the performance of biological polymers in the human body. His current research deals with silicone encapsulation of electrical stimulators implanted into the human body. The research was performed in the summer of 2005 at Tyndall National Institute in Cork, Ireland. In his spare time, George enjoys travel and art; though his free time has recently been greatly limited with the burdens of studying for the MCAT. George Desh is from Glenview, Illinois.

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Nirav N. Shah is a junior in the College of Arts and Sciences who is dual-majoring in environmental science and psychology while pursuing a pre-medical program. Born and partially raised in India, he has firsthand knowledge of how rapid development and industrialization impact the ecosphere. Previously, he has done research for NASA's Space Flight and Life Sciences Training Program. Currently, he is working on a research project with the Art Institute of Chicago in conjunction with Northwestern's McCormick School of Engineering to facilitate restoration work on Seurat's "Sunday at La Grande Jatte." He hopes to eventually apply his foundation in research and his anticipated medical degree as a member of the astronaut corps.

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Merina Thomas is a junior in the Weinberg College of Arts and Sciences and is completing a double major in biology and gender studies. She has enjoyed working in the Matoushek Laboratory since July of 2005. She has always been interested in molecular biology, and this project allowed her to delve into the biotech side of biological research crucial to improving experimental techniques in the future. She has plans to continue researching in the future and possibly automating SDS-PAGE gel loading with robotic gel loading or creating real-time fluorescence degradation assays for protein visualization. She would especially like to thank Dr. Matoushek and her mentor, Neil Jaffe, for all their support and guidance throughout the project.