DISCUSSIONS

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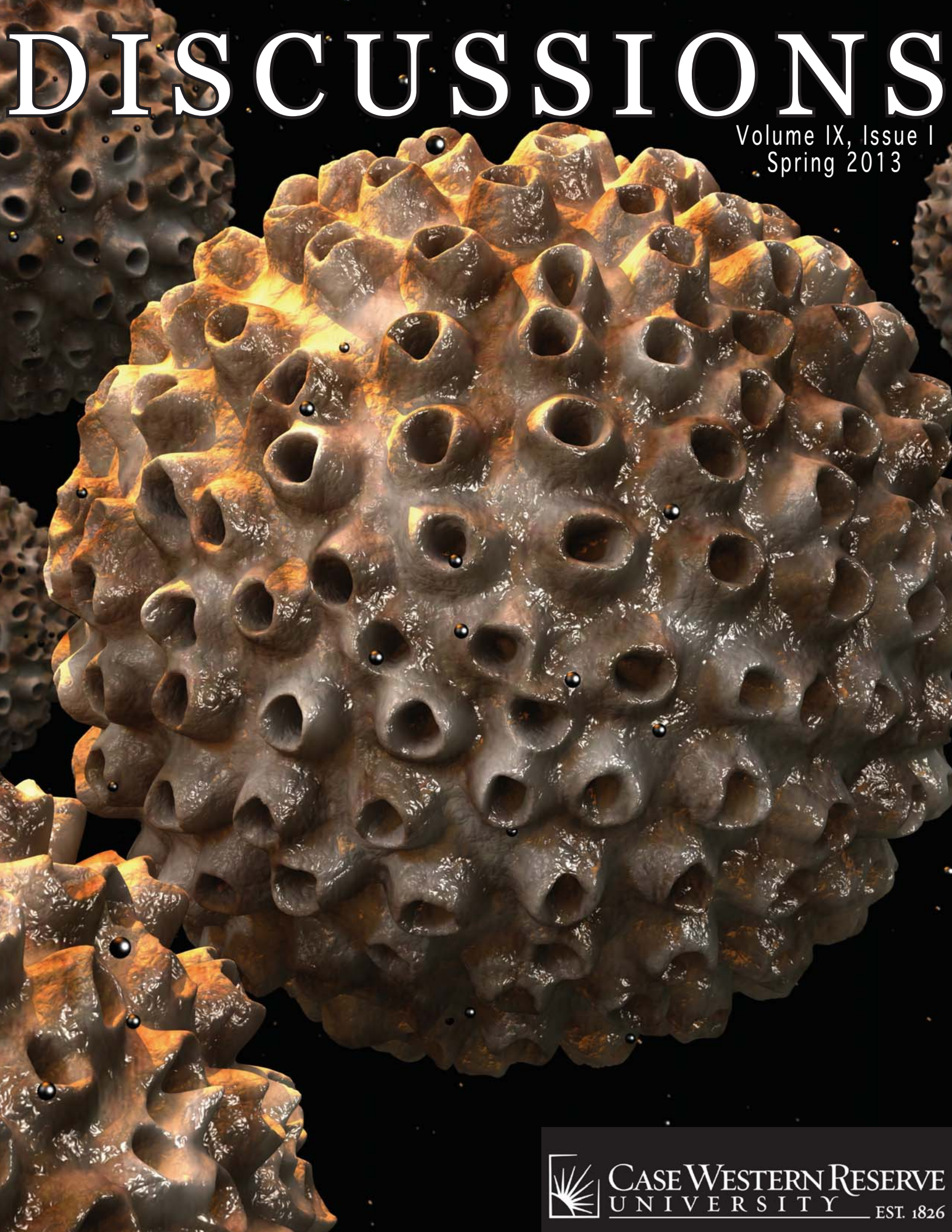

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DISCUSSIONS

The Undergraduate Research Journal of Case Western Reserve University

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Letter from the Editor

Dear Reader,

Thank you very much for opening up this journal and taking time out of your schedule to read Discussions. If you are unfamiliar, Discussions is an undergraduate peer-reviewed research journal based in Case Western Reserve University. Our main objective is to present the best of undergraduate research from across the country. It is my belief that current research is pushing the bounds of our knowledge and allowing our society to progress forward like never before.

The articles found in this journal were selected from a very competitive pool of submissions that we received at the beginning of the fall semester. Similar to the last issue, the rate of acceptance in this journal was 30%. The articles in this journal were among the very best and I would like to congratulate the authors on all of their hard work.

Discussions is in a time of growth and reconstruction. We are looking to expand distribution and readership of Discussions and we will need a lot of help and support. We are always looking for new staff members to bring aboard. Even by picking up this journal you are supporting the continued efforts of all the undergraduate research that occurs all over the nation.

If you would like to be a part of Discussions, there are several ways to help out. We have positions in review, editing, public relations, and design. If you would like to learn more about Discussions, feel free to email me at **nathan.kong@cwru.edu**.

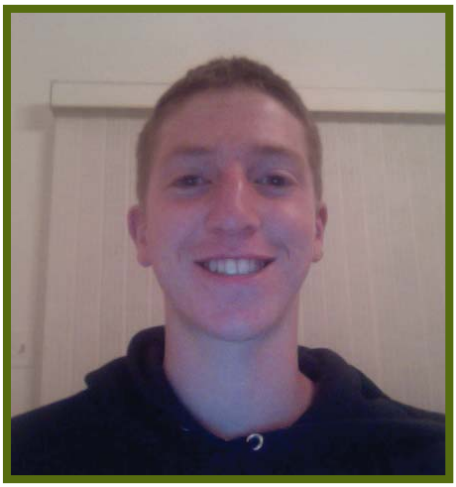
As a reminder, if you have new and exciting research and would like to be published in a peer-review journal, our next submission deadline is in **September 2013**. Submission guidelines and more information are located in this journal as well as on our website: **http://case.edu/discussions/**.

I would like to thank everyone who helped in the publication of this journal, including Media Board for being so supportive throughout the entire production. In addition, I would like to recognize Charles Fulco, our only senior. Charles has done a fantastic job over the past couple years getting involved with Discussions and doing everything from reviewing to laying-out and designing the journal. Finally, I would like to personally thank Sheila Pedigo, our advisor, as well as the entire SOURCE office. I hope you enjoy what you find inside and discover something new.

Sincerely,

Nathan Kong
Editor-in-Chief, Discussions Research Journal

Alginate aerogel/glass and carbon fiber composite substitutes for balsa cores in wind turbine blades



ABSTRACT

Aerogels are mesoporous solids composed of 90-95% air. Their remarkable properties include: high strength-to-weight ratios, low thermal conductivity, high absorptiveness, and tunable properties. In this experiment, five 5wt% (5 g of alginate for every 100 mL of deionized water) ice-templated alginate aerogels were prepared in a freeze-drying process. The samples were frozen either horizontally or vertically and then sandwiched between carbon fiber or glass fabric. The flexural moduli and specific flexural moduli were measured and will be compared to those of current wind turbine blade cores. Out of all of the samples prepared, and ignoring the samples with high uncertainty, the vertically frozen 1-0-1 aerogel structure reinforced with carbon fiber composite exhibited the highest flexural modulus (10.26 2.03 MPa) and the highest specific flexural modulus (3888 808.3 MPa/(g/mm^3)). This material may be able to serve as an environmentally-friendly substitute for balsa wood and synthetic foams in wind turbine blade cores.

INTRODUCTION

The global wind energy industry is growing and is set to make up a significant portion of the future electricity portfolio. A key area of wind turbine research and development focuses on blade materials. Until today, turbine blades were roughly the length of airplane wings and allowed for technology, computer software and material science to easily transfer from the aerospace industry to the wind industry. However, in the near future, newly manufactured blades will be much longer than airplane wings. This will cause aerospace technology to no longer be sufficient. This creates a high demand for new technology. More wind energy can be harnessed with longer blades; the power a turbine generates is proportional to the square of blade length. Longer blades, however, are heavier and experience more flap-wise and edgewise bending. . Blade weight is roughly the cube of blade length. This weight exerts more strain on the overall turbine structure. For these reasons, one of the most critical areas of wind energy innovation is in the manufacturing of high strength-to-weight ratio materials for turbine blades. Most blades today consist of an external fiberglass skin surrounding an internal layer, called the "core." The core is made of either PVC foam or balsa wood. The skin gives the blade flexural strength. The core reinforces the shell's flexural strength and provides shear and compressive strength. The material used in most turbine blade cores today is balsa wood. Balsa wood is a great material for the role because it has a high strength-to-weight ratio, is naturally available, and is biodegradable.

However, the global supply of balsa wood will not sufficiently satisfy orders for planned wind turbine development. This lack of supply creates a high demand in the wind-energy industry for new synthetic foams that can imitate the function of balsa wood in the blade cores. Leading foams include

Gideon Grossman

Gideon Grossman is a junior majoring in mechanical engineering at Princeton University. He is passionate about fighting climate change through clean renewable energy technologies and plans to work for a renewable energy company. In his free time he loves to rock out on the tenors in the Princeton University Band, swim, workout, and watch NOVA scienceNow videos.

Acknowledgements

Thank you to my Principle Investigator, Dr. David Schiraldi, my mentor Rocco Viggiano, and the rest of the Schiraldi group for their guidance and for answering my many questions with warm appreciation.Thank you to the National Science Foundation for its financial support. Thank you to the Great Lakes Energy Institute at Case Western Reserve University for organizing and running the REU SUR-WinD program this summer. It was an enjoyable research experience.

Table 1. Summary of samples prepared

Type	Fabric	Laminate Structure	Freezing Direction
A	None	0-0-0	Vertical
B	Carbon fiber	1-0-1	Vertical
C	Carbon fiber	1-0-1	Horizontal
D	Carbon Fiber	2-0-2	Vertical
E	Glass Fabric	1-0-1	Vertical
F	Glass Fabric	1-0-1	Horizontal
G	Glass Fabric*	2-0-2	Vertical

PVC G-foam and Corecell A-Foam. These foams have high strength-to-weight ratios, and are not in limited supply, but are petrochemical-based and their production processes release toxins. The latter traits make these materials violate the environmental ideals of wind energy. Alginate aerogels may serve as a new core material that, like balsa, are naturally available and biodegradable and, like the petrochemical-based foams, are in abundant supply.

Discovered in 1931, aerogels are recognized for their record-breaking properties including low thermal conductivity, high strength-to-weight ratios and high absorbency. Aerogels are mesoporous materials consisting of 90-95% air. They are used in aerospace equipment, construction, and packaging . The motivation behind this experiment was to find an aerogel/fabric composite that could replace balsa wood and PVC foam in the core of wind turbine blades. To qualify as an effective replacement, the material would need to exhibit a compressive, shear, and flexural strength, comparable to that of materials used in the core of wind turbine blades. If these alginate aerogel/fabric sandwiches exhibit satisfactory strength, they will serve as excellent blade materials for several reasons. The raw materials, algea and water, are in abundant supply; balsa wood is sparse. Alginate - readily obtained from algae - is nontoxic and biodegradable, and the freeze-drying process is cleaner than PVC production. The only waste product of this process is water vapor. Finally, aerogel properties are tunable, unlike those of balsa wood.

Seven different samples of alginate aerogel were prepared and tested for flexural strength. Each sample had a different arrangement of carbon and glass fiber. These fabrics were chosen for this experiment because of their high tensile strength and porosity. As mentioned above, strength is necessary for structural integrity of wind turbine blades. Porosity was of interest because it was hypothesized that porosity would enhance adhesion to the aerogel matrix by allowing alginate polymer chains to seep through the fabric pores. These fabrics were also chosen because they are commonly used in wind turbine blades.

EXPERIMENTAL

Materials

Ammonium Alginate, deionized water prepared using a Barnstead RoPure low pressure reverse osmosis system, glass fiber fabric (E-glass; 2 oz; 3 mil thick; made with an untwisted yarn; Sweet Composites, Bethesda, MD), and Carbon fiber.

Sample Preparation

Seven aerogel/fabric composite samples were prepared. One sample was prepared as a control with no fabric and was vertically frozen, three samples were made with carbon fiber, and three samples were made with glass fiber. Each set of three consisted of one 1-0-1 and one 2-0-2 laminate arrangement that was frozen vertically, as well as one 1-0-1 laminate arrangement that was frozen horizontally. Table 1 summarizes the various samples that were prepared.

This terminology will be explained further on in this portion of the report. Each sample contained a 5wt% alginate aerogel. The alginate was mixed with the deionized water in a Waring model MC2 mini laboratory blender for approximately 15 seconds, yielding a green, viscous, and gelatinous mixture. The gel was poured into a frame (the materials and dimensions of the frames used are described further on). The surface of the gel was evened out by sliding a precut plastic leveler over the gel. The sample was then freeze-dried in a Virtis AdVantage Model EL-85 (lyophilizer). The freeze-drying process consisted of two stages. In the first stage, the sample was frozen at a shelf temperature of -70 °C. A 6" x 6" frame with ½"-thick, 1" tall polypropylene sides and a ¼" thick aluminum bottom was used for the two 1-0-1 vertically frozen samples and the unlaminated zero-fabric vertically frozen sample. Due to the relatively high thermal conductivity of aluminum, versus that of polypropylene, ice crystals originated on the aluminum surface and grew in columns stemming perpendicularly outward from the aluminum surface, penetrating up through the gel. As depicted in Figure 1, as the crystals grew, they

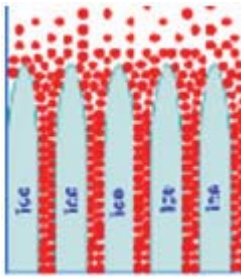


Figure 1. The blue portions denote ice. Reproduced with permission from Qian L. and Zhang, H.

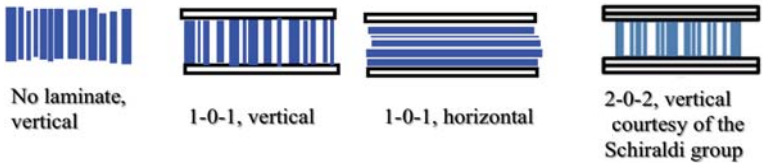


Figure 2: Laminate structures prepared

pushed the alginate polymer out of the way.

When an ice column encountered a chunk of alginate too thick to displace, the ice path skewed off to the side and connected to a neighboring ice column. This process arranged the polymer into a matrix formation, dubbed the "house of cards" structure.

In the second stage of the freeze-drying process, the lyophilizer was set to five microbars and room temperature (25°C). The sample was left in the lyophilizer at these conditions for three days until all of the ice had sublimed into water vapor. The final product was a strong, mesoporous material composed of connected columnar alginate fibers.

The 1-0-1 horizontally-frozen samples were prepared in exactly the same way as the 1-0-1 vertically-frozen samples, but in a different frame. The frame for horizontal freezing had two opposing aluminum sides, two polypropylene sides, and a polypropylene bottom surface. The goal of using this mold was to induce horizontal crystal growth. This was not the outcome, as will be discussed in the results section of this report.

The 2-0-2 vertically frozen samples were prepared in ½ " x 3" cutouts embedded in a 7" x 7" x 1" frame with polypropylene sides and an aluminum bottom.

The "1-0-1" samples were prepared for freezing by first laying a sheet of fabric on the bottom of the frame, then covering the fabric layer with 1 centimeter of gel, and finally covering the gel with a second layer of fabric.

The process used to prepare the 2-0-2 samples was similar to that used to prepare the 1-0-1 samples. The sole difference was that the sample was frozen in three steps instead of just one. First, a fabric/1mm of gel/fabric sandwich was placed in the freezer; just before that material fully froze, the sample was removed from the freezer and an 8mm-thick layer of alginate gel was poured on, followed by a third sheet of fabric. The sample was placed in the freezer a second time. Before the sample fully froze, it was taken out once more and a final 1mm-thick layer of alginate gel and fabric layer were added. The sample was placed back in the freezer for a third time. The purpose of this layer-by-layer method was to ensure that the fabrics remained even and the appropriate distances apart.

After the samples had freeze-dried, a band saw was used to cut them into uniform rectangular specimens matching ASTM standards (a width of 0.5" and length of 1"). The 2-0-2 samples did not need to be cut because they were prepared in molds that already had the appropriate dimensions of 0.5" by 1".

Characterization

A three-point bending test was performed on the specimens to determine their flexural moduli. Testing was done according to the ASTM-790 standard, using an Instron universal test machine with a 1mm/min crosshead speed and a 50mm span distance (Figure 3).

The exact length, width, and thickness of each specimen were measured with a digital caliper. The mass of each specimen was measured with an analytical balance.

Figure 3. Instron Universal Test Machine set up for flexural testing

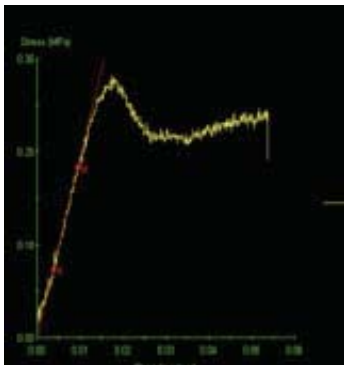


Figure 4: Stress-strain curve example (1-0-1 vertically-frozen, carbon fiber. Specimen #6)

Analysis

Load-displacement data from the testing machine was translated into a stress-strain curve and a flexural modulus was determined from the linear-elastic regime (Figure 4). The density of each specimen was calculated on Excel by dividing the mass by the volume. Volume was calculated by taking the product of the width, length, and thickness. Specific flexural moduli of each specimen were also calculated by dividing flexural modulus by density. For all samples, flexural modulus, density, and specific flexural modulus values of each specimen were averaged and standard deviations were calculated on Excel.

RESULTS and DISCUSSIONS

Flexural moduli, densities, and specific flexural moduli are presented in Table 2 and Charts 1 and 2. The 1-0-1 layered, vertically frozen glass fiber sandwich exhibited the highest flexural modulus and specific flexural modulus, but the value of each specimen in the sample were so varied (i.e. the sample's average values had high standard deviations) that the data was not significant. The sample that exhibited the highest flexural modulus (13.1 +/- 6.6 MPa) and specific flexural modulus (1.1E5 +/- 0.6E5 MPa/(g/mm^3)) with meaningful significance was the 1-0-1 vertically frozen carbon fiber sandwich (type B). Flexural modulus data for balsa wood was difficult to find in existing literature, so future work will include ordering a sample of SB. 100 balsa wood (the leading type of balsa in turbine blades) and testing it for flexural modulus in the same manner that the aerogel samples were tested in this experiment.

Aside from flexural strength, a turbine blade must also have high compressive and shear strength. Previously, Dr. Schiraldi's lab has tested the compressive strength and specific compressive strength of alginate aerogels of varied weight percentages with and without clay filler (Table 3). To expand upon this previous research, compressive and specific compressive moduli were looked up for balsa wood and leading synthetic foams (Table 4). The 15wt% with clay alginate aerogel sample had a specific compressive strength of 5.6E5 MPa/(g/mm^3). This value is nearly as high as that of balsa wood (6.1E5). This promising finding warrants further experimentation with alginate aerogels. The strength and specific strength can be further increased with the addition of cross-linkers and fibers, such as PVOH. The results in Table 3 also show that the increase in strength does not level off at the highest weight percentages prepared (15wt%). For this reason, a 20wt% sample was prepared and will be tested for both compressive and flexural strength. It should be noted that the 20wt% gel was difficult to pour evenly into the mold because of its high viscosity.

Table 2. Summary of results. *Will be retested because these samples had a large range of values

Type	Modulus (MPa)	Density(g/mm^3)	Specific Modulus
A	Not yet tested		
B	13.1±6.6	$1.2\times10^{-4}\pm0.1\times10^{-4}$	$1.1\times10^{5}\pm0.6\times10^{5}$
C	7.6±4.4	$7.2\times10^{-5}\pm2.8\times10^{-5}$	$9.7\times10^{4}\pm6.1\times10^{5}$*
D	10±2	$1.3\times10^{-4}\pm0.2\times10^{-4}$	$7.9\times10^{4}\pm2.5\times10^{4}$
E	$2.8\times10^{2}\pm3.0\times10^{2}$*	$1.9\times10^{-4}\pm0.2\times10^{-4}$	$1.4\times10^{6}\pm1.5\times10^{6}$*
F	5.9±0.9	$1.0\times10^{-4}\pm0.1\times10^{-4}$	$5.7\times10^{4}\pm1.5\times10^{4}$
G	Collapsed in freeze-dryer		

Chart 1: Bending Moduli (excluding the 1-0-1 verti-caly frozen glass sample glass sample

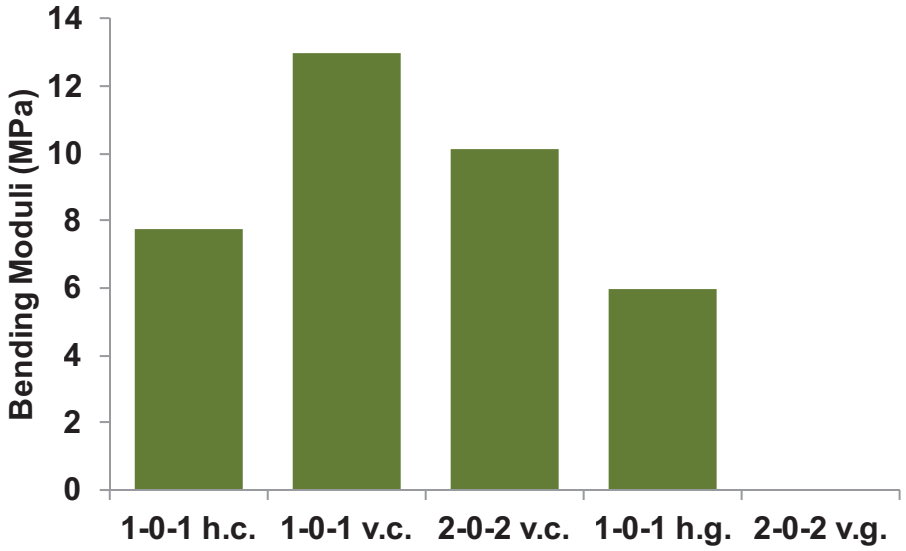


Chart 2: Specific Bend-ing Moduli (excluding the 1-0-1 vertically frozen glass sample)

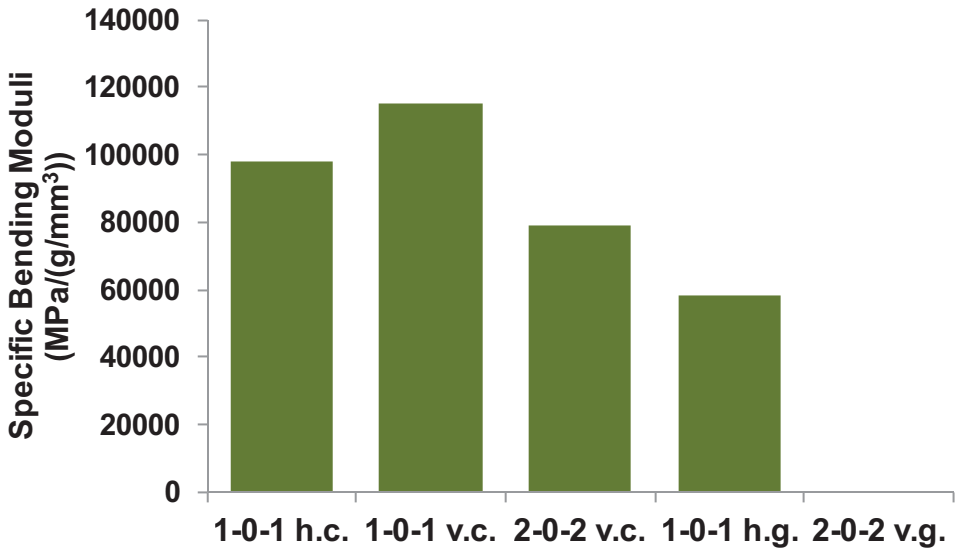


Table 3: Compressive Modulus, density and specific compressive modulus of alginate aerogels of varied weight percentages with and without clay. Reproduced with permission from Hongbing Chen. Modulus in MPa, Density in g/cm^3, Specific Modulus (M/d) in MPa- cm^3/g.

Alginate	Property	A5(5%)	A7.5(7.5%)	A10(10%)	A12.5(12.5%)	A15(15%)
No clay	modulus	0.99±0.06	3.2±0.2	9.4±1.4	20±2	46±9
	density	0.047±<0.001	0.066±<0.001	0.085±<0.001	0.108±<0.001	0.131±0.002
	M/d	21±2	49±3	110±17	185±16	350±64
C5 (5% clay)	modulus	5.8±0.7	21±3	42±5	70±7	97±11
	density	0.085±<0.001	0.108±0.002	0.130±0.002	0.152±0.002	0.174±0.002
	M/D	68±8	196±27	324±36	458±48	557±65

Table 4: Compression data for balsa wood and two leading synthetic cores. Balsa wood data source: Bech, A; Valsgaard P.. Foam data are from Gurit.

Material	Balsa Wood	PVCell G-Foam	Gorecell A-Foam
Compressive Modulus (Mpa)	460±71	65-300	41-217
Density (g/mm^3)	$7.5x10^{-4}$	$6x10^{-5}$ to $2x10^{-4}$	$6.9x10^{-5}$ to $2.1x10^{-4}$
M/d	$6.1x10^{5}$	$1.5x10^{6}$	$1.0x10^{-6}$

Another key observation was that the fabric layers on the top of the aerogel samples adhered to the aerogel well, while the fabric layer on the bottom did not adhere well. The supposed reason for this is that, while the gel was able to seep through the pores in the top fabric, it could not seep through the pores in the lower fabric because there was no room between the lower fabric and the frame's surface. In future experiments, this problem could be addressed by including a thin film of gel beneath the first layer of fabric.

Figure 5: The actual freezing direction of the 1-0-1 horizontally frozen samples.

Finally, a note about the directional freezing process: the samples that were supposed to freeze horizontally actually froze diagonally as depicted in Figure 5. The reason for this is believed to be that the distance from side to side of the mold is long enough to make it more likely for the ice crystals to encounter chunks of polymer, too thick to penetrate and cease growth in the horizontal direction.

Conclusions

With increased alginate weight percentages, and/or the incorporation of clay, fibers or cross linkers, alginate aerogels may be able to serve as a biodegradable and environmentally-sound substitute for balsa wood in turbine blade cores. The compressive strength of alginate aerogels with 15wt% and clay incorporation is nearly that of balsa wood and after the flexural strength of balsa wood is measured, a flexural strength comparison can be made as well. The results of this experiment warrant further research and may have profound implications for the developing wind energy industry.

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Human papillomavirus and Brazil: Critical study of prevalence and current situation in an epidemiological investigation

ABSTRACT

Human papillomavirus (HPV) is one of the most prevalent sexually transmitted pathogens associated with malignancy, infecting 12% of sexually active women, worldwide. HPV is also responsible for 99.7% of cancers of the cervix. Cytology and colposcopy are the recommended diagnostic methods by the Brazilian Ministry of Health and are the most frequently used tests during routine screening exams. In this regard, the Laboratory of Pharmacogenomics and Molecular Epidemiology (LAFEM) of the State University of Santa Cruz, Ilheus/BA (Brazil), performed a critical evaluation of HPV prevalence in women in the southern region of the state of Bahia, correlating with results of meta-analysis of data shown by DataSUS. The proposal was developed in partnership with the ongoing research in the Basic Health Units and in the Family Health Program. The statistical analyses were performed using the Meta-Analyst Beta 3.13 software. We observe that the estimate of the overall prevalence of HPV infection in women in Brazil to be 36.1%. Data of scientific literature showed that, in Bahia, the prevalence is 43.9%; our study presented prevalence of 47.7%. According PCR results, in the southern region of Bahia, cervical-cancer mortality has been increasing by 2.15% per year, since 2000. The findings denote fragility of the Health System regarding HPV prevention. Furthermore, this study highlights the close relationship between diagnosis and molecular methods, observed by the increase in sensitivity and specificity when applied with cytology. In the absence of strategies for surveillance, deploying relevant public actions in control and early detection of cervical cancer remain compromised.

INTRODUCTION

The Human papillomavirus (HPV) is a DNA virus member of the papovaviridae family, whose genes are located on the positive strand of viral DNA[3, 14, 19]. This large family of viruses is capable of infecting a variety of tissues, including the cutaneous epithelium and mucosa.

Since the early 1980s, the number of clinical and laboratory research publications regarding HPV has grown considerably, as has the use of molecular epidemiology and molecular biology in HPV research[7]. These advances in research allowed the discovery of the role of HPV – primarily HPV serotypes 16 and 186, 11 – as the etiological agent of cervical cancer.

Cervical cancer is a critical public health problem as it is the second most common cancer in women worldwide[6]. Although there have been advances in its diagnosis and treatment, cervical cancer still remains an important challenge in developing countries such as Brazil, where the incidence rate is twice as high as that of developed countries. In Brazil, 17,500 new cases of cervical cancer are expected by the end of the year, 2012[9, 10, 13].

The Papanicolaou smear test is the most widely used cervical cancer

Joao Do Nascimento

João Henrique Fonseca do Nascimento is an undergraduate researcher at Case Western Reserve University, and an undergraduate in baccalaureate of Biomedicine at State University of Santa Cruz (UESC), Ilheus – Bahia, Brazil. He worked on the epidemiology and prevalence of Human Papillomavirus (HPV) in women attending a Basic Unit of Women's Health, southern Bahia (Brazil). Currently, he is in the Center for Global Health and Diseases at Case, developing research in Genetics, Molecular Biology and Epidemiology, with Dengue virus, HCV and Schistosoma mansoni, working with Dr. Ronald Blanton.

Acknowledgements

I would like to thank my mentor, in Brazil, Dr. Sandra Rocha Gadelha Mello, for the opportunity and for advising me throughout this project; to Rafael Ponce Terashima, for the support in preparing the text. I also would like to thank the team of the Laboratory of Pharmacogenomics and Molecular Epidemiology (Brazil), the health professionals in the Health Units of the southern Bahia (Brazil), my colleagues; and to the Discussions team and to SOURCE for giving me the opportunity to present our research performed in Brazil.

Figure 1. Epidemiology of HPV in Brazil in 2012. The North and Midwest did not have enough data to generate reliable average values. (REGIONS: Green = North, Orange = Northeast, Yellow = Midwest, Red = Southeast, Blue = South).

Figure 2. Map of Brazil: 14 Brazilian states in blue where, from 2000 to 2011, there were no epidemiological publications on HPV available, according to the sources consulted.

screening test in the world. Organized screening programs reduces mortality due to cervical cancer. However, in developing countries, where screening programs are uncommon, the rate of death due to cervical cancer remains high. In this regard, recent studies have presented molecular diagnosticsas a great ally. Polymerase Chain Reaction (PCR) has been shown to be the most sensitive method for identifying HPV infection in clinical samples[17], and primers amplifying DNA fragments in the conserved L1 capsid antigen (L1) region have become the most widely used in clinical and epidemiological studies of HPV.

METHODS

We prospectively collected data of all women who presented themselves for routine cervical cancer screening at primary health care units (Unidades Básica de Saúde) in the Southern Region of the state of Bahia, Brazil. All women who agreed to participate provided written informed consent. Endocervical swabs were collected and sent to the Laboratory of Pharmacogenomics and Molecular Epidemiology (LAFEM) of the State University of Santa Cruz, Ilheus, Bahia – Brazil.All samples were analyzed by the Hematoxylin and Eosin (H&E) method as well as by Nested PCR. To perform the Nested PCR, we used primers amplifying DNA fragments in the conserved L1 region.. The MY09 and MY11 primer set-mediated PCR and the GP5+ and GP6+ primer set-mediated PCR are the most frequently used amplification systems for the detection of HPV DNA in clinical samples[17].

Staistical analysis

The data were analyzed according to the geometric theorem of annual growth rate for indicator analysis, pooled analysis, and analysis of variance. We used MetaAnalysis Beta version 3.13 software, developed by Tufts Medical Center and the Agency for Healthcare Research and Quality and Microsoft Excel 2010 software, developed by the Microsoft Corporation, USA.

RESULTS

During the period from July 2011 to April 2012, we performed the screening visits and the data analysis

for mortality due to cervical cancer in Brazil. Of the 195 women who participated in our study, 52 (or 26.7% of the sample) tested positive for HPV. We also observed that the sensitivity/specificity was 0.41/0.71, 0.7/0.805, and 0.909/0.783 respectively for Papanicolaou, PCR, and a combination of both methods (Fig. 4).

DISCUSSION

The data were processed obeying CI = 95% and p = 0.001. The study showed that Brazil has an average prevalence of HPV of 36.1 percent. The southern region had the highest prevalence of HPV infections and an average frequency of 43.9%. Mello and colleagues (2010) showed the southern region of Brazil to have one of the largest incidence rates of cervical cancer since the 1980s[12, 9, 20]; our results corroborate the Mello study's rates. Our study also showed that the regions of the Southeast and Northeast had average frequencies of 32.19% and 29.9%, respectively. The North and Midwest regions did not present enough data to generate reliable and significant values, with publications in the states of Amazonas[19, 5] and Goias[18] only. Additionally, our study showed a strong link between HPV and gynecological neoplasms, which has been already demonstrated as well.

According to the data of the National Control Program of Cervical Cancer and DataSUS, related to statistical calculations, mortality due to cervical cancer has been increasing by 2.15% per year in Brazil – one of the largest rates of any country around the world. Despite the high rate of frequency of HPV in the Northeast, the Women's Health Program (INCA) demonstrated in 2010 that in Bahia, the ratio of the sexually active female population and the number of cytological examinations required is relatively low, reaching only 14%, one of the lowest rates in the country. The available studies only evaluated HPV-HIV co-infection, and the population samples used were women not usually seen in the routine screening exams.

The link between national HPV prevalence and the noticeable lack of data is troublesome. Fourteen Brazilian states do not present reliable or relevant data about the prevalence of the virus over the last decade (Fig. 2). Moreover, a connection can be seen between the lack of data and the high cervical cancer mortality rate of young Brazilian women – approximately 2.16% – when compared with more developed nations such as the United Kingdom, which has a mortality rate of 1.3%[2]. In the United States, 4,008 women died from cervical cancer between the years 1999 and 2008; this death toll is similar to the annual number of deaths due to cervical cancer in Brazil during the same period (Fig. 3). This emphasizes the dependence of the viral control of HPV on sufficient data.

Another point that we can infer and discuss is the women's health care in Brazil, and questions of gynecologic malignancies. In early 2012, the National Institute of Cancer (INCA) published on their website "The actions of the

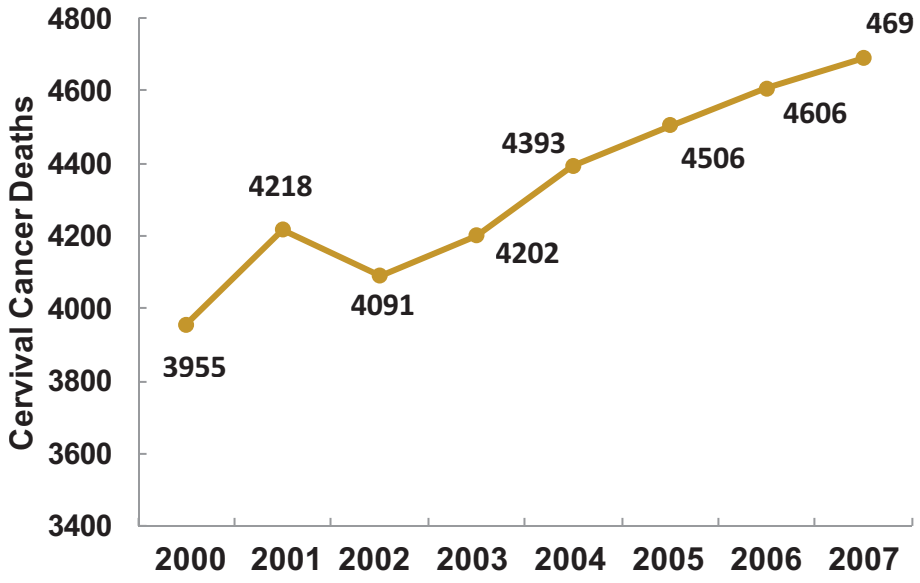

Figure 3: Deaths caused by cervical cancer during the years 2000 to 2007 in Brazil (SD = 257.9). Shows increasing rate of death of 2.156% per annum.

National Cancer Surveillance aim to know in detail the current framework of cancer in Brazil"[9, 10]; however, despite the strong link between HPV and cervical cancers already shown, there are no official epidemiological indicators in the system of public health in Brazil on the prevalence and frequency of this virus, despite the fact that it is the second-most common sexually transmitted infection in women attending primary care units in the country.

According to the World Health Organization, more than 80% of cases of cervical cancer occur in developing countries[21] such as Brazil, and according to United Nations Population Divisions in 2009, these countries contain 82% of the population of the world. This lack of consistent results on the magnitude of infections imposes limitations on the planning of public surveillance and control[8, 15].

We also discuss in this work, aspects related to the exams and tests for the HPV diagnosis used in Brazil. The Papanicolaou smear test is the exam used in gynecological routine that allows the visualization of lesions on uterine cervix. This procedure is used as a screening test for cervical cancer, which has a strong correlation with HPV infection - 99.7% of cases of Cervical Cancer are caused by HPV infection[12]. However, a Pap smear alone is not enough to perform an efficient diagnosis; for more accurate results, molecular methods are required. We observed in out meta-analysis studies, which showed that 15.6% to 20.2% of women may have negative cytology with positive viral load[15].

These results suggest the low accuracy and efficiency of the Pap test as a method of routine care, by itself. Therefore, molecular tests are important in giving an accurate diagnosis, and also have the advantage of possibly subtyping the virus, which can result in an effective treatment that will prevent the tumor formation (Fig. 4).

We also observed that sexual and health educations represent important roles in sexual transmitted infections, as HPV. In this order to control HPV infection and consequently lower the prevalence of cervical cancer, it is necessary to improve practices in sex education. Oria and Alvez, in 2004, conducted studies in guiding adolescents and their families in the face of sexually transmitted infections and to evaluate the role of health professionals. The results were alarming, demonstrating that dialogue between parents and children about sex, contraception, and condoms is defective or, in many cases, nonexistent. Despite the increasingly publicized that HPV is the most prevalent agent of cervical cancer in the world, there is a significant weakness in health education, which is shown by the high rates of infection and of deaths linked to cervical cancer. It is crucial to invest more in practices of sexual education, to work directly on the reality of knowledge of teenagers and their families. Thus, through direct and appropriate language, myths and taboos will be broken, opening space for the modification of the current landscape of the frequency of this severe infection

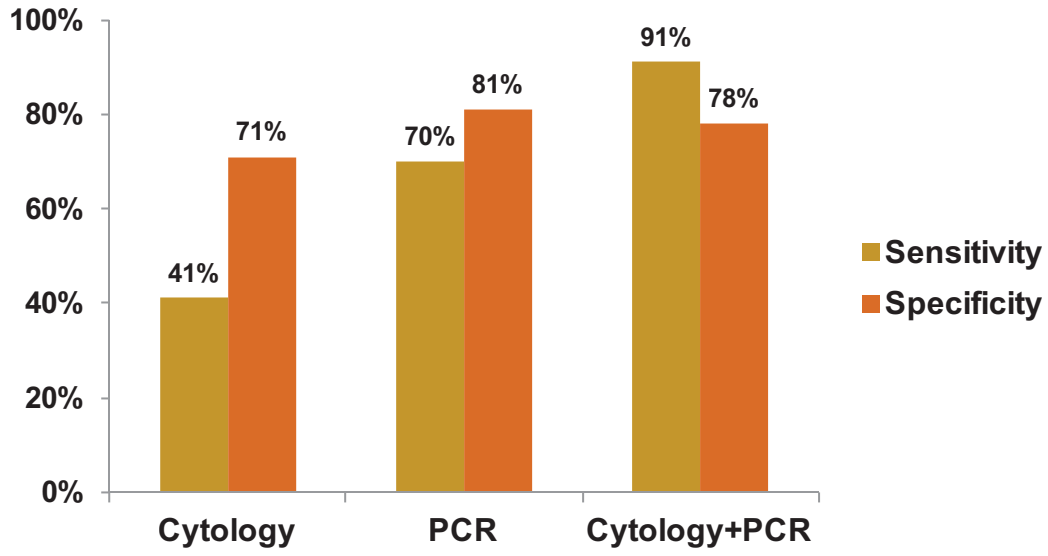

Figure 4. Evaluation of diagnostic tests for Human papillomavirus applied in the medical routine of the Brazilian public health system. In the Family Health Program of the Ministry of Health, the standard considered was hybrid capture. Analysis done by Meta-Analyst version 3.13 software.

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Optimization of combination reporter viruses for microscopy and cell sorting

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ABSTRACT

Using a novel combination reporter virus that allows monitoring of both HIV fusion and viral LTR-driven Enhanced Green Fluorescent Protein (EGFP) expression, an indicator of productive viral infection, we have found that over ninety-percent of the cells that undergo fusion with HIV do not become productively infected. Additionally, the extent of resistance to productive infection by HIV differs between immune cell subsets. We aim to understand the cellular genes and proteins that restrict productive HIV replication.

Currently, we are using an LSR II flow cytometer-based assay that detects CCF2-AM dye cleavage, indicating membrane fusion, and EGFP expression, which indicates productive infection. This allows assessment of the susceptibility of different immune cell populations to HIV infection. We seek to optimize the combination reporter virus for cell sorting and high-resolution microscopy, which will enable novel approaches to studying factors regulating the susceptibility of cells to HIV infection.

A major obstacle was encountered due to the fact that fluorescent proteins that work well on the LSR II may not be optimal for cell sorters and microscopes. Furthermore, the CCF2-AM dye is incompatible with EGFP, leading us to measure fusion and EGFP, in parallel. We aimed to develop a non-EGFP combination reporter virus that will be compatible on all machines. From a compilation of seven different dyes, we identified mCherry as a dye that worked well on all instruments except the FACSAria, which lacks a compatible laser for the red dye. In future work, we hope to test viruses carrying mouse CD24 protein, which is detectable with anti-CD24 antibodies in a variety of fluorescent colors and would be compatible with the FACSAria.

INTRODUCTION

Shortly following the discovery of the acquired immunodeficiency syndrome (AIDS) in 1981, the human immunodeficiency virus (HIV) was identified in 1983. Since then, much has been discovered about HIV, including the structures of many of its proteins and the basic mechanisms of attachment and fusion to cells. Despite all we know all about HIV, a cure has still not been developed. The problem with developing a single drug that will stop HIV infection is that the virus has an extremely high mutation rate and is, therefore, highly recombinant. HIV is classified as a retrovirus, which means after entering a cell, its RNA is reverse transcribed into DNA, after which viral DNA is integrated into the cell's genome. After integration, the cell produces the proteins necessary for the virus to form and bud from the cell membrane to infect more cells (Figure 1). Through the process of reverse transcription, errors are made in the sequence of the newly produced, double-stranded DNA due to the lack of a proofreading enzyme (HIV and Pathogenesis of AIDS). This

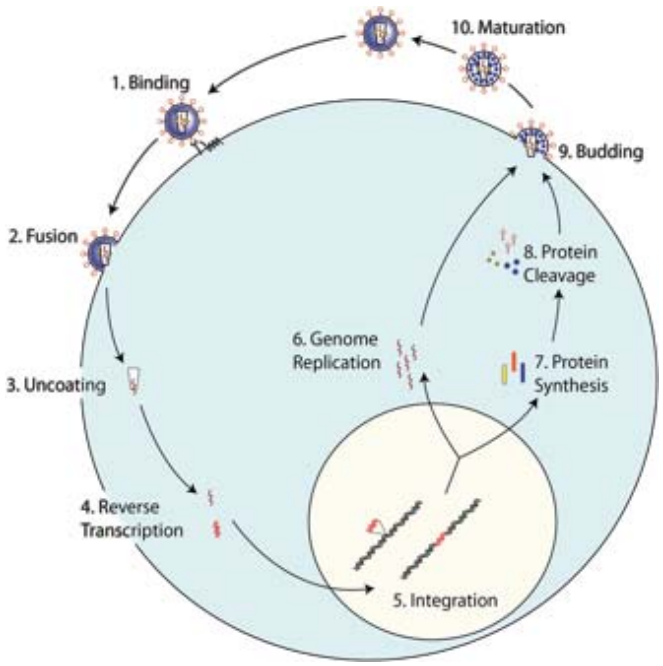

Figure 1. Viral life cycle. Assay measures fusion (step 2) and productive infection (step 7).

results in a tremendous diversity of viral sequences within a single infected individual. Thus, even when a drug works to stop certain strains of the virus, other strains of HIV are present in an individual that require different drugs to stop. Current drug therapies, therefore, consist of a cocktail of drugs that aims to target multiple stages of the viral life cycle simultaneously, slowing the emergence of resistance of virus.

While these therapies have been successful at slowing the progression of infection and extending the lifespan of patients, they do not cure the infection. A subpopulation of cells can become "latently" infected, wherein HIV integrates into the host cell's chromosomes, but does not produce viral RNAs or proteins. These latently infected cells are not sensitive to antiretroviral drug cocktails, allowing the virus to remain dormant for years. If a patient stops treatment, the virus inevitably returns and disease progression continues. Thus, lifelong therapy with antiretroviral medications is necessary.

Our laboratory is interested in the cellular factors that determine whether cells will become productively or latently infected once HIV has fused with the cell membrane. We are also interested identifying the exact subpopulations of immune cells that are targets for the virus.

Currently, we use a combination reporter virus assay that allows us to identify cells that have fused with HIV as well as determine whether or not these cells have progressed through the viral lifecycle to the point of viral protein production. By incorporating beta-lactamase (blam) into viral particles and loading target cells with the fluorescent blam sensitive substrate CCF2-AM, we can identify cells that have fused with HIV by observing changes in the optical properties of the substrate. When the virus fuses with and enters the cell, the beta lactamase in the viral particles reacts with the CCF2-AM dye present in the cells. This dye can then be excited at a specific wavelength, causing it to fluoresce, allowing us to determine if the virus has fused with the cells.

The most common fluorescent protein used is green fluorescent protein (GFP), isolated from the jellyfish A. victoria and from the sea pansy R. reniformis (Chalfie, 1995; Kar-Roy, et al., 2000). Since the discovery of this novel reporter protein, many others have been developed including dyes in red, blue, and yellow spectra (Ai, et al., 2007; Diéguez-Hurtado, et al., 2007; Marzylak, et al., 2007; Shaner, et al., 2005). These reporter proteins are used in HIV research as a marker for productive infection (Kar-Roy, et al., 2000). Because HIV is a retrovirus, it integrates itself into the cells' genetic material and utilizes the cells' machinery to produce more virus (HIV and the Pathogenesis of AIDS). Our reporter viruses also encode an enhanced green fluorescent protein (EGFP) gene driven by the viral LTR promoter, allowing us to identify in which viral proteins are being actively produced, an indicator for productive infection (Figure 1). In short, the cells that become productively infected will express a protein that fluoresces when stimulated at a specific wavelength. Using an instrument called an analytical flow cytometer (BD LSRII), we can identify subsets of CD4+ T cells that are susceptible to viral fusion and determine if they have become productively infected. The flow cytometer works on the principle of ordering cells into a single line through a column of solution. The dyes within the cells are then excited at certain wavelengths to induce fluorescence, which allows us to identify different substances present in the cell, depending on the dyes used. We aim to utilize this assay on machines other than the BD LSRII, including cell sorters (FACSAria and iCyt) and microscopes (Delta Vision). The cell sorter works similarly to the flow cytometer in that it can order cells and excite them. It can also separate the cells into subpopulations based on what wavelength at which it fluoresces. The flow cytometer allows us to characterize a sample, but opening up the assay to the other machines will allow for more in-depth analysis of our samples. For

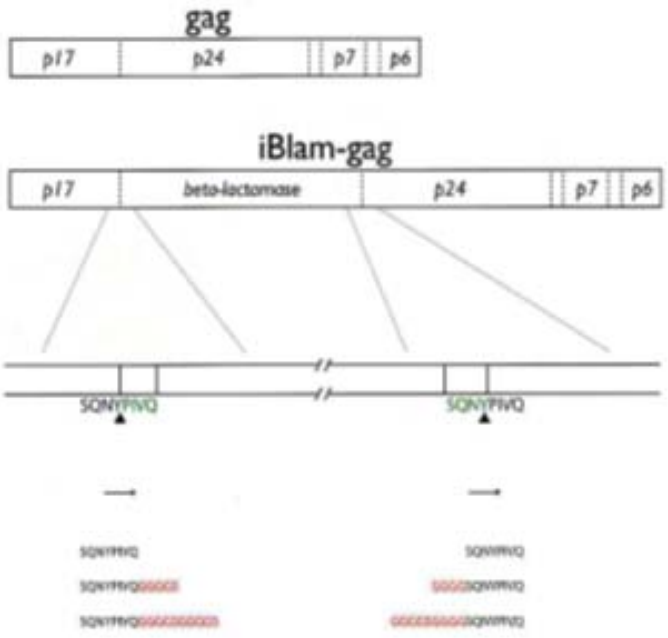

Figure 2. Beta-lactamase insert in the gag gene.

example, use of the cell sorter allows us to isolate populations of cells. Purifying these populations would provide the ability to analyze them by proteomics and RNA sequencing in order to identify factors regulating the progression of HIV through various stages of its life cycle. Furthermore, using this assay in conjunction with a microscope will allow us to see intact HIV target tissues, such as the lymph node or gastrointestinal tract, in order to better understand how HIV works in vivo. Ideally, we could adapt our assay for each machine and measure for CCF2-AM and EGFP, but every fluorescent dye and reporter gene has a specific wavelength of light that is optimal for excitation. Each dye and gene also has specific wavelength of light that is emitted upon energy release. One problem with our current assay is that the emission and excitation spectra of the CCF2-AM dye are extremely similar to that of EGFP, meaning that we cannot detect both simultaneously. Right now, we measure fusion using CCF2-AM on day one of infection and productive infection using EGFP on day 3 in order to avoid conflict, but in the future we hope to use a reporter gene other than EGFP in the assay so that we can measure fusion and productive infection simultaneously.

A second problem is that each instrument has different lasers and optical capabilities, so a reporter that works on the LSR2 may not be optimal–or even be detectable–on the other machines due to their unique laser configurations. This leads us to the purpose of this experiment: to evaluate which dye would be most compatible with each machine. We tested seven different dyes (EGFP, mCherry, Katushka, tagRFP, tagBFP, EYFP, and Cerulean) on each instrument. Our results suggest that mCherry is the ideal candidate for the adaptation of our current fusion and productive infection assay. It works well on all machines except the FACSAria, which lacks a suitable excitation laser. Further research may aim to test viruses encoding a mouse CD24 gene that is expressed similarly to EGFP and that is detectable with anti-CD24 antibodies available in a variety of fluorescent colors.

Figure 3. Instrument capabilities. *DeltaVision Microscope uses 7-channel LED illumination source, not lasers as the other instruments use.

Laser	LSRII	FACSAria	iCyt	DeltaVision Microscope*
355nm	x		x	
405nm violet			x	
407nm violet	x	x		
488nm blue	x	x	x	
561nm yellow-green	x		x	
633nm red	x	x		
640 HeNe			x	
UV	x			

The second part of this experiment was to construct a viral genome where the beta-lactamase gene is inserted into the gag region of HIV (Figure 2). In the current version of our combination reporter virus assay, we use a viral genome with beta-lactamase inserted into HIV's Vpr gene (blam-Vpr). Unfortunately, Vpr is only incorporated into HIV at about 50 to 100 molecules per particle. If we could insert blam into the Gag gene, it would be incorporated into the viral particles approximately 5,000 times, a 50-fold increase from where we are now. An increase in the amount of beta-lactamase present in virions would provide two significant advantages. First, it would allow us to detect cells that have undergone fusion with HIV for longer periods of time, possibly leading to simultaneous detection of fusion and reporter gene expression (using a reporter gene other than EGFP). Second, higher levels of blam in the viral particles would shorten the duration of time needed to see conversion of the CCF2-AM dye, potentially allowing us to monitor viral fusion in real-time. This is critical in microscopy of tissue specimens that typically have limited viability *ex vivo*.

Figure 4. Fluorescent reporter protein excitation and emission spectra.

Fluorescent Protein	Excitation Max (nm)	Emission Max (nm)
tagRFP	555	584
mCherry	587	610
Katushka	588	635
Cerulean	433	475
EGFP	488	509
EYFP	514	527
tagBFP	402	457

Hypothesis

According to the excitation maximums and the instrument optical capabilities, it appears that EGFP on the 488nm blue laser would be best suited for these three machines. To simultaneously measure fusion and productive viral infection, however, we need a different florescent protein. Any of the other proteins presented here may work well, but none will be optimal because 488nm laser is the only one that all three machines have. Therefore, each machine will not be capable of exciting any one dye, except EGFP. Additionally, none of the lasers exactly match up to the excitation maximums of the available proteins. Empirical testing of the fluorescent reporter genes will be required.

METHODS and MATERIALS

We began by growing up DNA from glycerol stocks through transformation in bacteria. A 500uL stock was set up and, after a 24-hour incubation period, was transferred to a 5mL stock and was left to incubate for another 24-48 hours. Upon completion of the incubation, a Qiagen plasmid purification kit was set up to isolate the plasmid DNA from the bacteria. The DNA isolated by this process was then used as an HIV core in combination with a viral envelope and blam-vpr DNA in a transfection with 293T cells. These cells incubated over a period of a few days to allow for ample production of the virus. The virus harvested from the transfection was then used to infect CD4+ T cells

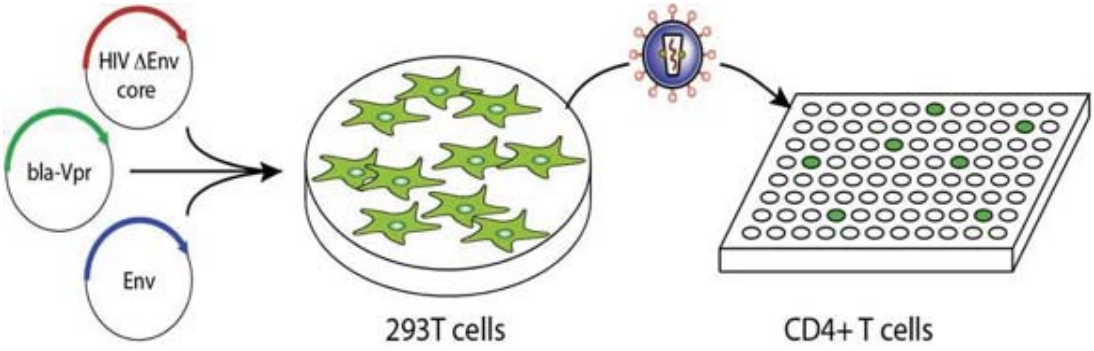

Figure 5. Pseudovirus production. Transfection and spinfection.

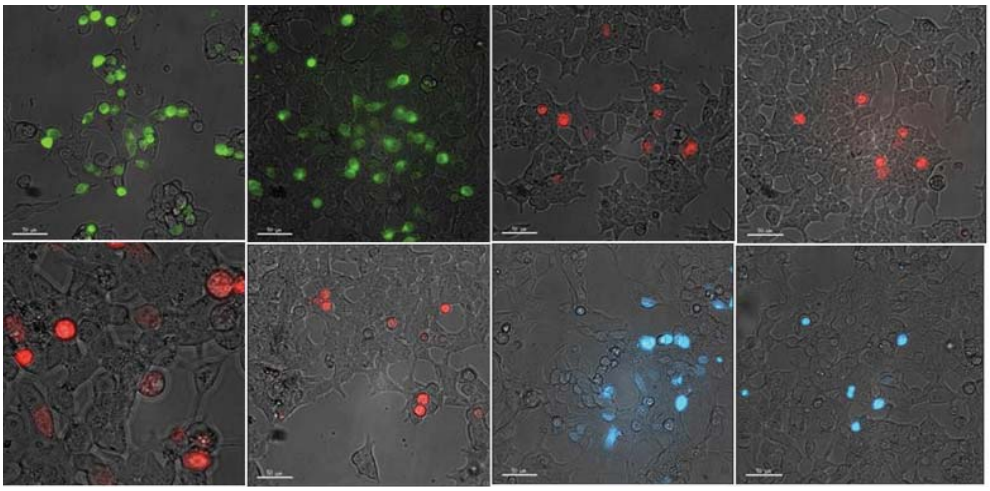

Figure 6. Images from DeltaVision microscope. Top row from right to left: EGFP day 1 after transfection, EYFP 1 day after transfection, Katushka day 1, and mCherry day 1. Bottom row from right to left: tagRFP(11) day 2, tagRFP(23) day 1, tagBFP day 2, and Cerulean day 2.

through spinfection. The CD4+ T cells were then left to incubate for a few days, and subsequently analyzed on an instrument such as the LSRII or FACSAria.

To work towards our goal of a better beta-lactamase construct, we used a series of PCR cloning techniques that would allow us to link each segment of our product (the gag p17 region, beta-lactamase, and the gag p24 region). Our aim was to fuse these three segments together and then introduce them into an HIV core vector using restriction enzyme digestion. Constructs were designed introducing blam to the p17 region and the p24 region with no linkers, one linker, and two linkers. Linkers were segments of nucleic acids that introduced space between the blam and p17 and p24 region respectively. These were used to allow more space for conformational shapes of the proteins formed. This was to ensure that the protein had enough room to get into their proper conformations.

RESULTS

All fluorescent proteins worked well on the DeltaVision microscope (Figure 6). EGFP and EYFP had the best rates of productive infection, but the brightness of all proteins worked well. EGFP and EYFP worked comparably well; all red dyes worked comparably well; and tagBFP worked better than Cerulean.

For LSRII data, the red peaks indicate the fluorescence emission of an untransfected cell population while the blue peaks indicate cell populations expressing

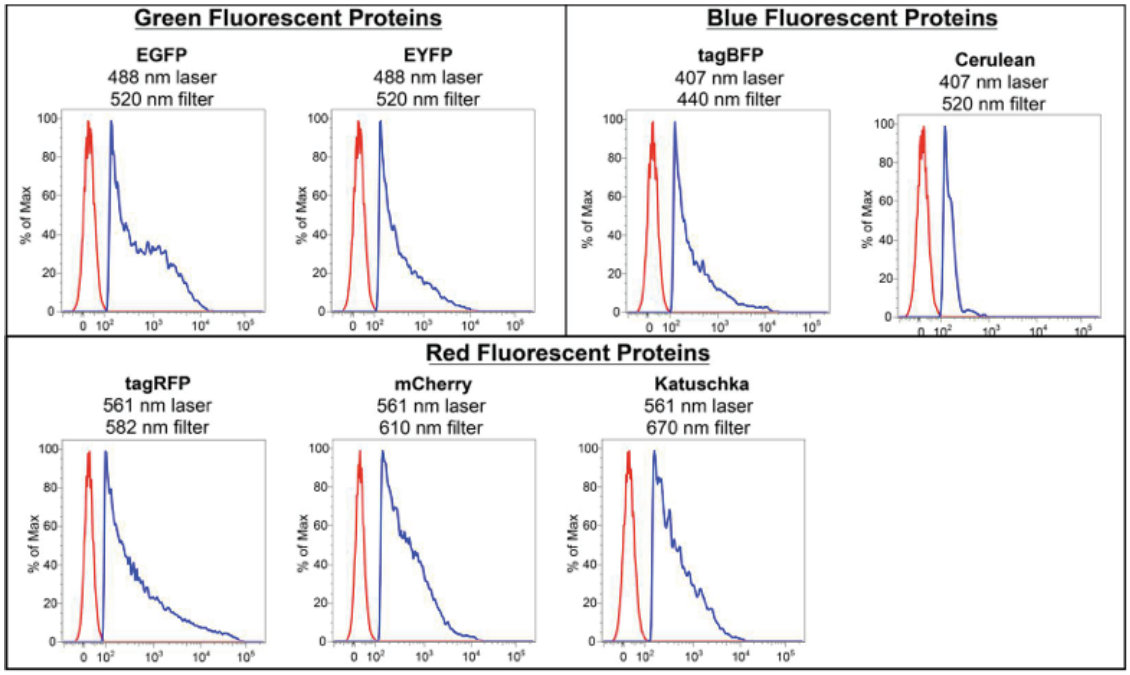

Figure 7. Untransfected cells (red) and cells expressing the fluorescent proteins (blue) utilized in our reporter viruses.

the reporter construct (Figure 7). EGFP worked better than EYFP; tagBFP worked better than Cerulean; and mCherry worked the best out of the red dyes, with Katushka as the second best of the red dyes. Overall, mCherry was the optimal dye for the LSRII, with what was quantitatively the strongest signal.

For the second part of the experiment, we successfully constructed cloning intermediates p17-blam and blam-p24 through overlap PCR, but were unsuccessful in generating a full-length p17-blam-p24 clone. We altered the annealing temperature and extension time, but could not get the desired product. Our next attempt was to try adding all three segments at once, which was successful, but we were unsuccessful in cloning the products into the backbone vector. After a series of attempts at the PCR process, we tried a different method, called Gibson Assembly, but those attempts were also unsuccessful. The Gibson Assembly Method required each segment of desired DNA as well as primers to be combined in a single isothermal reaction. The failures in cloning are likely due to the complex nature of this cloning project, the large size of the HIV core vector (14+kb), and the tendency of HIV to recombine.

CONCLUSIONS

From an array of seven unique dyes, we identified mCherry as a dye that worked well on all instruments except the FACSAria, which lacks a laser with an appropriate wavelength to excite red dyes. In future work, we hope to test viruses carrying the mouse CD24 gene. As with florescent reporter genes, mCD24 is produced following uncoating, reverse transcription, integration of HIV into the host genome, and viral LTR-driven gene expression. Unlike fluorescent reporter genes, the mCD24 molecule is not itself fluorescent, but is detectable with anti-CD24 antibodies that can be obtained in a variety of fluorescent colors. Several of these colors are compatible with the FACSAria cell sorter.

Furthermore, determining why the cloning of the beta-lactamase gene into the gag region of the viral genome was unsuccessful will hopefully lead to the development of a method that will allow the clone to work.

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