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Editors: Inquiry: The University of Arkansas Undergraduate Research Journal

THE UNIVERSITY OF ARKANSAS
UNDERGRADUATE RESEARCH JOURNAL



INQUIRY

Volume 19

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FOREWORD

I am pleased to present to you Volume 19 of the *Inquiry Undergraduate Research Journal*. *Inquiry* provides a forum for sharing the research and creative endeavors of undergraduate students and their faculty mentors at the University of Arkansas. The *Inquiry Journal* was developed by the Teaching Academy of the University of Arkansas and is supported financially and conceptually by the offices of the Provost and the Vice Provost for Research and Economic Development. The *Inquiry Undergraduate Research Journal* website, online publications, and journal archives are expertly managed by the David W. Mullins Library staff.

Volume 19 of the *Inquiry Undergraduate Research Journal* features the unique contributions of undergraduate student authors and their faculty mentors. The research and creative endeavors that are published in the *Inquiry Undergraduate Research Journal* span diverse fields at the University of Arkansas, including Biological Sciences; Landscape Architecture; Economics; Mechanical Engineering; and English. Turning to the research studies in this issue, Jessica Darby, Economics, explores the ways in which rice future prices respond to news releases by the USDA. Bailey Deloney, Journalism, conducted a content analysis to examine whether television commercials on Nickelodeon that target children favor traditional gender stereotypes. Sarah Plavcan, English, examines Milton's poem, *Paradise Lost*, to keenly investigate the conflict between predestination and free will. Griffin Sonaty, Biological Sciences, examines the use of loop-mediated isothermal amplification (LAMP) to detect single genes from bacteria in blood serum samples to aid in sepsis diagnosis. Scarlett-Marie Acklin, Biological Sciences, examines THz imaging and spectroscopy as an innovative method for detecting breast cancer tissues of patients with tumors 2mm or larger. Clint Paul, Mechanical Engineering, use SMD simulations to study the mechanisms associated with heterotrimeric collagen, when interacting with HAP under shear and peeling loading conditions; findings could have potential applications in engineering and medicine.

I would like to extend a special thank you to the many faculty members who volunteer their time and expertise in order to mentor the student authors and the faculty members who provide comprehensive reviews of student manuscripts. While we are unable to publish all of the submitted manuscripts, we also want to thank the students and faculty mentors for their diligent efforts. Additionally, I would like to thank Dean Carolyn Allen, Beth Juhl and Dylan Hurd, David W. Mullins Library, for their efforts in publishing each volume of the *Inquiry Undergraduate Research Journal* electronically. Please see the next page for a list of faculty and staff who play an integral role in publishing each volume of the *Inquiry Undergraduate Research Journal*.

We plan to publish Volume 20 of the *Inquiry* journal in March 2016. I encourage undergraduate students and faculty mentors to consider submitting their manuscript to the *Inquiry Undergraduate Research Journal* by May 16, 2016 for consideration.

Marcia A. Shobe, Editor

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Information Content of USDA Rice Reports and Price Reactions of Rice Futures

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Abstract

Rice is a predominant food staple in many regions of the world, and it is important to determine how efficiently the U.S. rice market helps to ensure world food security. This question can be answered by gauging the price discovery performance of the U.S. rice futures market and the economic usefulness of the U.S. government's supply and demand forecasts. So, to this end, we employ two event study approaches: (1) to examine variability in returns on report-release days as compared to returns on pre- and post-report days, and (2) to regress price reactions on changes in usage and production information. It is found that the USDA provides the rice futures markets with valuable information and rice futures respond to the information in an economically consistent manner.

Introduction

Rice is a predominant food staple in many regions of the world, and international rice markets play a vital role in ensuring the food security needs of developing countries. The U.S. rice industry has an important role to play in feeding the world's population. The U.S. is the 5th largest rice exporter, accounting for approximately ten percent of world trade, and compared to other crops, a large portion – approximately 45% – of rice produced in the U.S. is exported. So an important question to address is how efficient is the U.S. rice marketing system in meeting these world food needs? Specifically, how valuable is the U.S. rice futures market and supply and demand information published by the U.S. Department of Agriculture (USDA) to the U.S. rice marketing system? There is significant evidence that futures markets play a vital price discovery role in the U.S. grain marketing system for raw commodities such as corn and soybeans, and that the main source of economic trading information used to guide production and marketing decisions for the U.S. grain industry are government reports provided by the USDA. These reports comprise U.S. and World Agricultural Supply and Demand Estimates (WASDE), National Agricultural Statistics Service (NASS) Crop Production forecasts, and NASS Prospective Plantings and Acreage estimates. The actively traded corn and

soybean futures markets have been found to adjust quickly to supply and demand information contained in these government reports and to provide important pricing signals to farmers and the grain industry (Adjemian, 2012; Garcia, Irwin, Leuthold, & Yang, 1997; Isengildina-Massa, Irwin, Good, & Gomez, 2008a; Isengildina-Massa, Irwin, Good & Gomez, 2008b; McKenzie, 2008; Sumner & Mueller, 1989).

However, no prior research has attempted to analyze the economic value of USDA rice forecasts and the price discovery role played by the U.S. rice futures market. The U.S. rice futures market is relatively thinly traded compared with other grain futures, which begs the question as to how efficiently this market is able to discover price by embodying relevant economic information. In addition, the U.S. rice market exhibits a number of production-based idiosyncrasies making it somewhat unique compared with other grains. So, for example, the potential economic value of USDA production forecasts may be less for rice compared with other grains because it is an irrigated crop with much lower production variability (McKenzie, 2012). With this in mind, the overarching aim of this study is to estimate the economic value of these reports in terms of their impact on U.S. rice futures prices and their ability to support the U.S. rice industry's production and marketing decision-making.

Methodology

USDA Methods and Procedures

In this section, we provide a brief description of the three types of USDA reports and the data contained in these reports that we examine in our empirical analysis. First, the NASS Crop Production reports project forthcoming harvest-time rice supply based upon forecasted acres, yield, and overall production. These reports are released monthly from August through November.

The second type of USDA report that we consider consists of the WASDE reports, which are released each month and which provide forecasts of beginning stocks, imports, production, domestic food, industrial, and seed use, residual use, exports, and ending stocks over each crop year. The U.S. rice harvest typically occurs over the September through October period while the crop year runs from August to July of the following year; as a result, WASDE reports contain both pre- and post-harvest information about rice markets. We focus attention on WASDE reports released in May through November. The May through July reports use statistically based model forecasts from historical and expected data while the August through November reports utilize the NASS Crop Production report information.

The third type of USDA report is the NASS March Prospective Plantings report, which estimates planted area based on a survey of producers (representative stratified sample) completed during the first two weeks of March.

It is important to note that WASDE and NASS Crop Production reports are released simultaneously during August through November and the crop supply numbers are identical in the two reports. Therefore, in our empirical analysis, we are in effect considering the impact of the Prospective Plantings report in March, the impact of WASDE reports alone in May through July, and the joint impact of WASDE and NASS Crop Production reports in August through November.

The releases of all monthly WASDE reports for rice from January 1990 to December 2014 were analyzed in this study. Within this time period, a total of 287 WASDE reports were released; October 2013 is the only month without a report release due to the government shutdown. The WASDE report is typically released between the 9th and 12th of the month, but the

time of release varies across the sample period. From January 1985 to April 1994, monthly reports were released at 3:30 p.m. EST, following the close of the Chicago Board of Trade (CBOT) trading session. From May 1994 to December 2012, with the exception of December 1994, monthly reports were released at 8:30 a.m. EST, prior to the start of CBOT trading session. From January 2013 to current, monthly reports were released at 12:00 p.m. EST, during the CBOT trading session. Additionally, March Prospective Plantings reports, which are typically released between the 28th and 31st of March, were analyzed for the same time period and had the same change in release time.

For futures prices, Chicago Board of Trade opening and closing futures prices for current (at the time of release) year rough rice November contracts were collected for six trading days prior and five trading days following the release of the WASDE, NASS Crop Production, and Prospective Plantings reports. While previous studies have used the nearest-to-maturity contracts for each release to measure price reactions to information about current market year information, November contracts were used in this study to capture price reactions to forthcoming market year information. The November futures contract is the harvest-time new-crop contract in rice markets. As such, it is the first contract to cover the forthcoming market year and should be sensitive to market expectations about harvest production and beginning stock levels for the forthcoming market year. In addition, the November contract is highly liquid, both in terms of volume and open interest, as it is used to hedge future levels of expected production over the pre-harvest period.

Event Study Approach

A vast literature has explored if various USDA Crop Production reports contain new and unanticipated information. Typically, an event-study framework is utilized to test whether significant changes in market prices occur following the release of a report. Event studies are based on the premise that information is valuable to the market if prices react to the release of reports (Campbell, Lo, & MacKinlay, 1997). If reports contain only anticipated information at the time of release, then futures prices will not react and the report does not provide “news” to the market. The underlying assumption is that markets are not strong form efficient, only semi-strong form efficient, as futures prices would

reflect both public and private information in a strong form efficient market (Fama, 1970). Thus, markets would already anticipate the information contained in the USDA reports. The concept of futures market efficiency and informational content of USDA reports are intrinsically linked in the event-study approach (McKenzie, 2008).

Sumner and Mueller (1989) examined the informational content of USDA reports and its impact on corn and soybean futures prices. The impact was measured by the absolute mean differences between futures price changes following the report release and futures price changes on non-report release days. Sumner and Mueller concluded that USDA reports provided news to the market, as the absolute mean price change following the release of the report was higher than that of non-report release days. Similarly, Isengildina-Massa et al. (2008a) examined the impact of WASDE reports using the change between the closing price immediately prior to the report release and the opening price immediately following the report release for corn and soybean futures contracts over the 1985-2006 time period. Known as the close-to-open return, this price change captures the impact of information made available between those two points. The results of the event study found that WASDE reports have a substantial impact on corn and soybean futures markets, as illustrated by a return variance on report releases almost three times that of pre- and post-report return variances. Thus, there is information provided in WASDE reports that is unanticipated in corn and soybean markets. Fortenbery and Sumner (1993) employed a similar methodology and compared close-to-close futures price and option premium returns on report release days to returns on non-report release days. The results of their analysis, however, found that the report releases from 1985-1989 did not result in larger average returns. “One cannot rule out that USDA reports still provide news, but that the news can no longer be measured by a simple price change variable” (Fortenbery & Sumner, 1993, p.172).

If traders’ perceptions of supply and demand are altered by the release of a WASDE report, then this new information should be reflected in a change in futures prices (Fortenbery & Sumner, 1993). As such, November rough rice futures prices represent the market’s expectation of rice prices at harvest time. Variability of futures price returns around report releases including market news should “spike” upon announcement and

maintain normal pre-report variability levels for days following the announcement. The “spike” reflects a change in the market’s expectation of prices due to the news included in the announcement.

Following Isengildina-Massa et al. (2008a), the time index for this event study is $t = -6, \dots, -1, 0, +1, \dots, +5$. In order to account for the change in release times over the course of this sample period, $t = 0$ indicates the trading session at the CBOT immediately following the release of a WASDE (or equivalently NASS Crop Production report), or Prospective Plantings report. For WASDE reports, the event index is $i = 1, \dots, 287$, where $i = 1$ represents the January 1990 release of the WASDE report and $i = 287$ represents the December 2014 release of the WASDE report. For Prospective Plantings reports, the event index is $i = 1, \dots, 24$, where $i = 1$ represents the March 1990 Prospective Plantings report and $i = 24$ represents the March 2014 Prospective Plantings report.

If WASDE, NASS Crop Production, or Prospective Plantings reports include news, the information should be reflected in futures price movements immediately following the release of the report. For this reason we analyze the change or return in future prices based upon the percentage difference between closing futures prices observed just prior to report release and opening futures prices observed immediately after report release. Closing prices represent the average prices traded at the end of a day’s trading session while opening prices are representative of the first trades in a session. For the statistical tests in this study, variances of close-to-open returns were analyzed to investigate the reaction of rough rice futures prices. The close-to-open returns for a given WASDE (NASS Crop Production) or Prospective Plantings report release date were calculated as:

$$(1) \quad r_{t,i} = \ln(p_{t,i}^o / p_{t-1,i}^c) \times 100$$

$$t = -6, \dots, 0, \dots, +5,$$

where $p_{t,i}^o$ is the opening price of the current (at time of release) year November rough rice futures contract for session t and event i , $p_{t-1,i}^c$ is the closing price of the current (at time of release) year November rough rice futures contract for session $t - 1$ and event i , and \ln is the natural logarithm. The natural logarithm is used to measure daily percentage returns, which allows us to estimate and compare price reactions across years when rice traded at vastly different price levels.

A one-tailed F-test was applied to close-to-open returns. The null hypothesis is that the return variability for the report release session is less than or equal to the variability of the pre-report and post-report sessions. The alternative hypothesis is that the return variability for the report release session is greater than the variability of the pre-report and post-report sessions. Pre- and post-report session returns were aggregated to obtain a single estimate of non-release day session variances across the different reporting months. This is referred to as pre/post session variance in subsequent results sections. All statistical tests were computed using Data Analysis Toolpak included in Excel software.

Private Forecasts and Regression Analysis

Regression analysis can also be utilized to determine if various USDA Crop Production reports contain new and unanticipated information. Typically, the regression model involves regressing futures price changes on a dummy variable for report release dates as well as other explanatory variables (Fortenbery & Sumner, 1993). The regression-based event study model is typically estimated using Ordinary Least Squares (OLS), or using Weighted Least Squares (WLS), in the presence of heteroskedasticity (Fortenbery & Sumner, 1993). The estimated regression coefficient measures the average price response to a change in the news provided in USDA reports. Fortenbery and Sumner (1993) regressed the futures price change against loan prices, U.S. share of the world markets, and availability of option markets.

In order to compute the magnitude of news included in USDA reports, an estimate of market expectations measured just prior to USDA report release dates is necessary to capture the anticipated versus unanticipated component of USDA reports. As noted earlier, and according to the Efficient Markets Hypothesis, futures markets should only react to new unanticipated information. Due to the growth of private firms providing information on agricultural markets, a number of studies (Egelkraut, Garcia, & Good, 2003; Garcia et al., 1997; Good & Irwin, 2006) have utilized private information to proxy the amount of anticipated information in the market. Theoretically, the price impact of USDA production forecasts should be determined by how well the market anticipates the forecasts (Good & Irwin, 2007). Good and Irwin (2006) found that, on average, USDA corn production forecasts were more accurate than private market forecasts for most

of the 1970-2003 time period. However, production forecast errors for USDA and private firms were highly correlated, suggesting that the private market anticipates at least some of the information in USDA reports. Thus, unanticipated information is measured as the difference between private information and information contained in USDA reports.

Alternatively, Lehecka (2014) assumed that the crop-condition information in Crop Progress reports serves as a proxy for anticipated information, thus the unanticipated information component is reflected in a change in crop-condition information from one report to the next. The study examined the relationship between changes in information provided in USDA Crop Progress and immediate price reactions. A WLS procedure was utilized, and close-to-open returns on report release sessions were regressed against the difference in the percentages of the crop in excellent or good condition from week to week. The results of the study indicated that there are price impacts from unanticipated information, and that Crop Progress reports provide significant informational value to corn and soybean markets.

There is a distinct lack of rice forecasts and information supplied by private analytical firms. This is in stark contrast to the large amount of private forecasts and information provided to other grain markets. Thus, in our analysis, the production and usage information included in the previous (at the time of release) USDA reports are used as a proxy for anticipated information. So, similar to Lehecka (2014), the news or unanticipated information is measured as the difference in production and usage numbers from one USDA report to the next. This measure of “news” allows us to empirically test if there is a private information gap in rice markets between the releases of public USDA numbers. If rice futures prices react to changes in month to month changes in USDA numbers, this would indicate that (1) any interim private information does not fully replace or adjust market expectations based upon previous month USDA numbers; and that (2) any interim private information does not fully foreshadow the information contained in newly released USDA numbers.

A typical event study model can be written as an Ordinary Least Squares (OLS) regression:

$$(3) f_{+1} - f_{-1} = \alpha + \beta (F_{ijt}^{USDA} - F_{ijt}^{Private}) + e_t$$

where $f_{+1} - f_{-1}$ represents the price change from the closing November futures price on the day prior to the report release to the opening November futures price on

the first trading day after the report release. The term, $F_{ijt}^{USDA} - F_{ijt}^{Private}$ represents the “news” element of USDA reports, where F_{ijt}^{USDA} represents the USDA forecast of either usage or production i , observed in month j and year t , and $F_{ijt}^{Private}$ represents the private market consensus forecast of either usage or production i , observed in month j and year t . And e_t is a mean zero normally distributed error term.

In the traditional event study approach, the estimated regression coefficient \hat{b} measures the average price response to a one-percentage point change in the “news” element of USDA reports. Thus, it is assumed that futures prices only react to the element of USDA production forecasts that was not anticipated by the private sector.

For this study, the previous month USDA reports serve as a proxy for private production forecasts, as it is assumed that the private production forecasts contain no additional information compared to previous month USDA forecasts. In this case, equation (3) can be rewritten as:

$$(4) \quad f_{+1} - f_{-1} = \hat{\alpha} + \hat{b} (F_{ijt}^{USDA} - F_{i,j-n,t}^{USDA}) + e_t$$

where the private forecast is replaced with the previous USDA forecast observed at time $j-n$. This would be the USDA forecast observed in the previous month and the term $F_{ijt}^{USDA} - F_{i,j-n,t}^{USDA}$ would measure the monthly revision in USDA forecasts as in (Isengildina, Irwin, & Good, 2006).

$$(5) \quad f_{Nov} - f_{jt} = \hat{\alpha} + \hat{b} (F_{iNovt}^{USDA} - F_{ijt}^{USDA}) + e_t$$

Equation (5) above was used to analyze USDA forecasts observed at time j compared to the USDA forecast observed in November, which has final numbers for production and usage. In this case, the term $F_{iNovt}^{USDA} - F_{ijt}^{Private}$ would measure the USDA forecast error for a specific month (at the time of the report) in comparison to final estimates published in November. In this case, F_{ijt}^{USDA} represents the USDA forecast of harvest area, yield or usage i , observed in month j and year t , and F_{iNovt}^{USDA} represents the corresponding final USDA estimate of harvest area, yield or usage i , observed in month j and year t .

Results

Event Window Returns

The impact of USDA reports on event returns is illustrated graphically in **Figure 1** for close-to-open return variances for March, May, June, July, August, September, October and November. With the exception of September, the overall pattern of return variances for each of the months is consistent with the prediction that the return variance “spikes” on release days. The most notable statistically significant release “spikes” occur in July and October. For July, close-to-open report return variance is approximately 2.2 times larger than the pre- and post-report return variances. For October, the close-to-open report session return variance is approximately 3.6 times greater than the pre- and post-report return variances.

Table 1 presents the close-to-open F-test statistics for pre- and post-report return variances as compared to report session return variance. With the exception of September, there was substantially higher variance on report sessions than on pre- and post-report sessions when observing close-to-open prices. If markets overreact to the USDA report releases, then the close-to-open returns could overstate the “news” component included in the report (Isengildina-Massa et al., 2008). However, close-to-open returns tend to best represent the instantaneous incorporation of new information into market prices, and other information events, observed over the course of the release date trading day.

One would expect to see large reactions to July and August information, as these represent the first release times of truly new production information. Subsequent USDA reports might contain less unanticipated supply information, hence less variability for September. October may be more variable as the market adjusts to final (i.e. “more certain”) production numbers. Adverse weather events, such as too hot nighttime temperatures over summer months, which are known to adversely affect rice yields, are over by October, and the effects are now realized and reflected in October production numbers for the first time. For this reason, the futures returns during the September period may be particularly noisy across the whole event window.

These explanations for the F-test results should be qualified by the fact that reports contain projected usage information as well as projected supply

Figure 1. Pre-Report, Release, Post-Report Session Close-to-Open Return Variances¹
(January 1990-December 2014)



Table 1: Rice Futures Close-to-Open Return Variance Test Results for WASDE Reports
(January 1990-December 2014)

Month	Report Session Return Variance	Pre/Post Report Return Variance	F-Statistic
March	1.569	0.701	2.240***
May	1.826	0.929	1.963***
June	1.256	0.975	1.288
July	1.763	0.803	2.195***
Aug	1.618	0.777	2.083***
Sep	0.885	0.815	1.086
Oct	2.953	0.814	3.630***
Nov	1.412	1.252	1.128

*Indicates significance at the 10% level,
**Indicates significance at the 5% level,
*** Indicates significance at the 1% level

estimated coefficients in **Table 2** are statistically significant and of the expected sign. An increase in production, a supply side factor, should elicit a price decrease – as indicated by the negative coefficient. An increase in usage, a demand factor, should elicit price increase – as indicated by the positive coefficient. The results illustrate that futures price responses to supply and demand information across the pre-harvest period are consistent with a well-functioning and efficient market. Prices rise with respect to increased usage, or demand shocks, and fall with respect to increased production, or supply side shocks. Furthermore, as indicated by the R² in **Table 2**, changes in production and usage information account for a larger proportion of overall variation in futures prices movements later in the season. September and October have the highest R² as any changes in production and usage information account for more of the variation in futures prices movements as it gets closer to harvest. A battery of residual diagnostic tests, presented at the foot of **Table 2**, indicates that our model is well specified.

The average rice futures price over the time period of January 1990 to December 2014 was \$9.78/cwt. The results in **Table 2** show that, on average, a 1 percent unanticipated increase in production from the July to August period would elicit, on average, a 0.49 percent decrease in futures prices, a decrease of 5 cents/cwt. Conversely, a 1 percent unanticipated increase in usage from the July to August period would result in, on average, a 0.57 percent increase in futures prices, an increase of 6 cents/cwt. On average, unanticipated changes in production elicit the largest futures price responses from the September to October period at -0.71 percent, a decrease of 7 cents/cwt. On average,

¹The following dates did not have available opening futures price data and were excluded from the event windows for the close-to-open return variance results: April 6, 2012; November 4, 2012; November 8, 2012; November 12, 2012; November 14, 2012; March 24, 2013; March 25, 2013; March 26, 2013; May 7, 2013; May 8, 2013; May 9, 2013; May 14, 2013; May 15, 2013; May 16, 2013; May 17, 2013; May 20, 2013; May 21, 2013; November 7, 2013; November 10, 2013; November 12, 2013; November 13, 2013; November 14, 2013.

information. Usage shocks can be revealed at any time during the year, so the spike in October numbers could have also been the result of large unanticipated change in usage numbers over the sample period. The regression analysis results in this study will be able to offer more discerning results as to the relative impacts of both supply (production) and demand (usage) shocks revealed in this report.

Regression Analysis – Price Reactions to Month to Month Pre-Harvest Information

Regression results for immediate futures price reactions to production and usage “news” announced in USDA reports across the pre-harvest period from June to November are reported in **Table 2**. With the exception of the July usage coefficient, all of the

unanticipated changes in usage elicit the largest futures price responses from the October to November period at 0.94 percent, an increase of 9 cents/cwt.

On a month-to-month basis, the change in production and usage numbers announced in USDA reports elicits an immediate futures price response; public “news” drives futures prices in rice markets. Irwin, Good, Gomez, & Isengildina (2002) found that futures prices only respond to news unanticipated by private forecasts, which are released prior to the official public USDA numbers. The results in **Table 2** suggest that private rice forecasts released between monthly USDA reports are not viewed by rice futures markets as complete information updates on previous USDA report numbers. The fact that rice futures prices react to these month to month changes in USDA numbers indicates that any interim private information does not fully foreshadow the information contained in newly released USDA reports.

Table 2: Futures Price Reactions to Production and Usage “News” Announced in USDA Reports across the Pre-harvest Period

Parameters	June	July	Aug	Sep	Oct	Nov
Constant	0.29 (0.26)	0.19 (0.46)	0.20 (0.28)	-0.44 (0.17)	-0.17 (0.25)	-0.46 (0.28)
Production	-0.38* (0.19)	-0.20* (0.11)	-0.49*** (0.17)	-0.44*** (0.09)	-0.71*** (0.14)	-0.43* (0.26)
Usage	0.87** (0.33)	0.21 (0.19)	0.57** (0.26)	0.27** (0.11)	0.54*** (0.18)	0.94*** (0.27)
R ²	0.19	0.07	0.21	0.51	0.50	0.30
Q (1)	0.37 (0.55)	3.96 (0.05)	1.55 (0.21)	0.24 (0.62)	0.00 (0.91)	0.00 (0.97)
Q(2)	0.55 (0.76)	4.17 (0.12)	2.56 (0.28)	0.42 (0.81)	2.43 (0.30)	1.71 (0.43)
LM(1)	0.39 (0.53)	3.64 (0.06)	1.49 (0.22)	0.38 (0.54)	0.01 (0.91)	0.10 (0.76)
B-P	1.69* (0.43)	1.12 (0.57)	1.32 (0.52)	3.40 (0.18)	1.16 (0.56)	0.75 (0.69)
F Test	3.79* (0.04)	1.91 (0.17)	4.27** (0.03)	13.64*** (0.00)	12.51*** (0.00)	5.94*** (0.01)

*Indicates significance at the 10% level,

**Indicates significance at the 5% level,

*** Indicates significance at the 1% level

White standard errors are presented for regressions with heteroskedasticity

Regression Analyses

Price Reaction to Information Changes from Pre-Harvest Time Periods to Harvest Time

Regression results for futures price changes regressed on harvest area, yield, and usage forecast errors of USDA reports across the pre-harvest period from June to November are reported in **Table 3**. All of the estimated coefficients in **Table 3** are statistically

significant and of the expected sign. An increase in harvest area and production, supply side factors, should elicit a price decrease – as indicated by the negative coefficient. An increase in usage, a demand factor, should elicit price increase – as indicated by the positive coefficient. Prices rise with respect to increased usage, or demand shocks, and fall with respect to increased harvest area and yield, or supply side shocks. Furthermore, as indicated by the R² in **Table 3**, changes in production and usage information account for a large proportion of overall variation in futures prices movements throughout the pre-harvest period. This is in contrast to the month-to-month results in **Table 2**, which indicated that the regression explains more as harvest approaches. Once again, residual diagnostics show that our model is well specified.

As mentioned previously, the average rice futures price over the time period of January 1990 to December 2014 was \$9.78/cwt. The results in **Table 3** show that, on average, a 1 percent unanticipated increase in harvested area from the July to harvest period would elicit, on average, a 3.13 percent decrease in futures prices, a decrease of 31 cents/cwt. Additionally, a 1 percent unanticipated increase in yield from the July to harvest period would elicit, on average, a 3.33 percent decrease in futures prices, a decrease of 33 cents/cwt. Conversely, a 1 percent unanticipated increase in usage from the July to harvest period would result in, on average, a 2.63 percent increase in futures prices, an increase of 26 cents/cwt. On average, unanticipated changes in harvested area elicit the largest futures price responses from the September to harvest period at -3.17 percent, a decrease of 31 cents/cwt. On average, unanticipated changes in yield elicit the largest futures price responses from the June to harvest period at -3.34 percent, while unanticipated changes in usage elicit the largest futures price responses from the October to harvest period at 4.53 percent.

The price of U.S. rice is highly influenced by the export market, as almost half – about 45 percent - of domestic production is exported (Childs & Livezy, 2006). The extent to which, if any, domestic supply influences futures prices is an open question. The results in **Table 3** suggest that domestic supply factors (i.e., yield and harvested area) do influence futures prices across the pre-harvest period.

Table 3: Futures Price Changes Regressed on USDA Forecast Error of Harvest Area, Yield and Usage Measured at USDA Report Times across the Pre-harvest Period

Parameters	May	June	July	Aug	Sep	Oct
Constant	1.23 (4.62)	2.07 (5.28)	5.28 (3.22)	3.48 (2.39)	2.23 (2.64)	1.11 (1.77)
Harvest Area	-2.68*** (0.78)	-2.45** (1.00)	-3.13*** (0.91)	-2.84*** (0.70)	-3.17*** (0.80)	-2.01*** (0.77)
Yield	-3.21*** (1.23)	-3.34** (1.41)	-3.33*** (0.88)	-3.29*** (0.66)	-2.85*** (0.71)	-2.07*** (0.75)
Usage	2.99*** (1.03)	2.95*** (1.16)	2.63** (0.94)	2.86*** (0.82)	3.07** (0.03)	4.53*** (1.55)
R ²	0.42	0.31	0.44	0.58	0.43	0.33
Q (1)	0.00 (0.91)	0.00 (0.79)	0.16 (0.69)	0.00 (0.79)	0.12 (0.72)	0.00 (0.91)
Q(2)	0.79 (0.68)	0.94 (0.62)	0.72 (0.70)	1.78 (0.41)	3.11 (0.21)	1.06 (0.59)
LM(1)	0.33 (0.56)	0.41 (0.52)	0.23 (0.63)	0.10 (0.75)	0.15 (0.70)	0.05 (0.83)
B-P	10.75** (0.01)	9.35** (0.03)	4.92 (0.18)	8.50** (0.04)	5.41 (0.14)	10.93** (0.01)
F Test	6.70*** (0.00)	4.54** (0.01)	7.17*** (0.00)	11.98*** (0.00)	7.04*** (0.00)	4.83** (0.01)

*Indicates significance at the 10% level

**Indicates significance at the 5% level

*** Indicates significance at the 1% level

White standard errors are presented for regressions with heteroskedasticity

Discussion and Conclusion

The purpose of this study was to determine whether USDA reports, specifically WASDE, NASS Crop Production, and March Prospective Plantings, reveal valuable “news” information to the U.S. rice industry. If reports contain only anticipated information at the time of release, then futures prices will not react and the report does not provide “news” to the market. Two event study approaches were utilized to: (1) examine variability in returns on report-release days as compared to returns on pre- and post-report days, and (2) regress price reactions on changes in usage and production information.

The most notable release “spikes” occur in July and October, as reflected in the close-to-open variances. The regression analysis indicates that the supply (harvest area and yield) shocks have the largest impact on July variances. This is consistent with the fact that July is the first release time of truly new production information, as the June Prospective Plantings data are incorporated in the July WASDE report. By October, the market has adjusted to final production numbers with more certainty, but demand shocks can be revealed at any time. Thus, the regression analysis indicates that the impact of demand (usage) shocks have the largest impact on October variances.

From a practical standpoint, our regression results show that the USDA provides the futures market with important information, which is vital to the price discovery process. The pre-harvest information and futures price reactions are correlated, thus futures prices are driven by new information as it is released. Private rice forecasts released between monthly USDA reports are not viewed by rice futures markets as complete information updates on previous USDA report numbers due to the fact that rice futures prices react to these month-to-month changes in USDA numbers. Any interim private information does not fully foreshadow the information contained in newly released USDA reports, thus suggesting that there is an information gap between USDA reports. The information gap is an opportunity that could be profitably exploited by private firms that could provide accurate and timely forecasts of monthly USDA numbers.

This is the first event study to measure futures price reactions to USDA reports in rice markets, and the findings are significantly different than that of event studies on corn and soybeans. Studies (Good & Irwin, 2006) in corn and soybeans find that futures prices only respond to news unanticipated by private forecasts. On a month-to-month basis, corn and soybeans futures prices do not react based solely on information provided in USDA reports. There is a large amount of private information that is incorporated in the reactions.

Due to the lack of private forecasts, rice futures prices tend to react to information provided in USDA reports more than corn and soybeans futures prices. While rice is sensitive to many of the same market factors as corn and soybeans, rice markets have a number of idiosyncrasies and are very different from other grain markets. All rice is irrigated, thus yields are much less variable. Production is sensitive to availability of water resources and some weather variability, but overall production risks are lower. Furthermore, rice markets have thinly traded futures markets. In fact, the Risk Management Agency is not providing 2015 Crop Year rice revenue protection coverage because there is not enough trading volume in November (harvest time) contracts.

Production and usage information is necessary to attract speculative interest in futures contracts and to aid in the price discovery process. Pricing signals from the futures markets are important for all participants in the supply chain – from farmers to exporters to retailers to consumers. Futures markets cannot discover price

in an information vacuum – futures markets need to trade based on comprehensive and frequently published supply and demand information (McKenzie, 2012). If the production and usage information provided to the marketplace can be improved and the error in USDA’s pre-harvest forecasts reduced, then movements in rice futures could be better predicted. Rice futures price realignments over the pre-harvest period indicate that more timely and accurate private forecast of production and usage could potentially be used to exploit trading strategies. Future research is needed to determine the extent of profitability of private forecasts in rice markets. Furthermore, future research could consider developing and testing trading strategies based upon advanced knowledge of information contained in USDA reports or upon private forecasts released prior to USDA reports.

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**Polly Pocket & Ninja Turtles:
A Content Analysis of Gender Stereotypes in Children's Advertisements**

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Abstract

This content analysis examined the use of gender stereotypes, in the forms of product association and various behaviors traditionally expected of a particular gender, in children's advertisements aired on Nickelodeon network. In spite of the current trend of Femvertising and successful campaigns such as Always's #LikeAGirl, results of this study revealed that although children's commercials appear to be breaking away from some long-standing gender stereotypes, many stereotypes remain. These stereotypes can be damaging to a child's self-esteem, self-view and self-realization. We find that commercials on Nickelodeon favor boy characters in overall time on-screen while girls-only commercials made up the lowest percent of advertisements in the sample. Additionally, children's commercials reinforce the social expectation that boys play outdoors with construction and transportation toys, while girls play indoors with dolls and stuffed animals. Enduring behavioral stereotypes include the idea that boys are competitive and aggressive, while girls are nurturing and domestic.

Introduction

Children represent a vast and increasing consumer market in the United States. They are purchasers, as well as major influencers in the buying of goods and services (Bakir, Blodgett, & Rose, 2008; Marketing Charts, 2014). Small children and pre-teens are estimated to influence more than \$1.2 trillion a year in direct purchases (Horovitz, 2011; Robinson, 2012). According to a 2014 Nielsen report, children have a particularly significant influence in the industries of music, books, home entertainment and video games (Nielsen, 2014). In fact, over half of 2014's top-selling DVD/Blu-ray titles were targeted toward child audiences, or were based on young adult fiction or comics (Nielsen, 2014).

In 2014, children ages 2-11 watched nearly 23 hours of television per week; children ages 12-17 watched an average of 19 hours (Nielsen, 2015). Strasburger and Hogan (2013) reported that children today spend more time with media than in school. In light of this, advertisers recognize the crucial role that children play in the consumer market, and as a result, they carefully design a large portion of their marketing communications with this specific target audience in mind. A significant share of this budget is dedicated to television commercials.

By nature, children tend to be vulnerable, naïve, and easily swayed, due to their lack of experience and incomplete cognitive development (Reynolds, 2011). Given that children have limited processing abilities, the Children's Advertising Review Unit (CARU) cautions advertisers to take into account kids' "special vulnerabilities" and "susceptibility to being misled or unduly influenced" (2009, p. 3). The extent to which advertisers adhere to the guidelines of CARU is a question researchers have examined for decades (Whipple & Courtney, 1980).

Principle 6 from the CARU guidelines (2009, p. 5) states: "Advertisers should avoid social stereotyping and appeals to prejudice". Although advertisers' primary objective is to sell the product by *reflecting* society in commercials, stereotypes also *shape* society and the way that children perceive themselves and others. Given that children's mental reasoning capabilities are not fully developed, they are often unable to discern the persuasive intent or unintended negative messages that stereotypes may carry in advertisements (Reynolds, 2011). The "absorbing" and "fascinating" nature of television can serve as a powerful depiction of negative stereotypes circulating within society (Mitu, 2011, p. 917). This contrast presents viewers with the real difficulty of discerning fact versus fiction. Though a 30 second commercial may seem of little importance in the course of a person's day, the repeated content and

underlying messages presented in advertising impact society. For example, gender stereotypes can inhibit the development of children and limit the scope of their self-realization, thus impeding the growth of society as a whole.

However, it should also be noted that television programs and commercials can serve as a conduit to present positive typecasts and also reinforce a society's culture and values. For example, "Fem-vertising", pro-female advertising that presents women in roles which challenge traditional gender biases, is a recent and quickly increasing movement within the advertising industry (Mitchell, 2015). The Cannes Lion Festival, an event that USA Today calls the "Academy Awards of Advertising", added an award called 'the Glass Lion: The Lion for Change' to recognize the most outstanding efforts in communications to portray gender equality (Mitchell, 2015; Toure, 2015).

In light of the various gender expectations and representations prevalent in advertising, this study investigates the behavioral patterns and gender stereotypes, as well as the negative messages that may be imbedded in those stereotypes that advertisers present to children today. In this study, researchers examine the implied association between gender and a range of behavioral variables, such as competitiveness, nurturing, aggression, athleticism, dancing, independence, parallel behavior and cooperativeness, many of which fall under the categories of character behavior and character interaction.

Review of the Literature

Social cognitive theory purports that characters in television commercials offer child-viewers models of behavior (Gilmore & Jordan, 2012). This suggests that television has the potential to impact viewers' beliefs and attitudes about social norms (Mastro & Stern, 2004). By providing models who display socially accepted behavior, television can influence the behavior of viewers themselves. Along the same lines, distinctiveness theory predicts that children will pay closer attention to and become more influenced by advertisements featuring characters that they perceive to be similar to themselves (Gilmore & Jordan, 2012). Social identity theory posits that people "derive much of their identity from association with groups" (Comello, 2011, pp. 313-314). Thus, if children identify themselves to be part of a certain group, such

as a particular gender, they may be more likely to try to align their behavior or appearance with that group.

Collectively, these theories suggest that children who see models of their own gender in television advertisements may alter their own attitudes or behavior in accordance to the characters that are presented to them (Comello, 2011; Gilmore & Jordan, 2012). Thus, advertisers, and the media in general, have a powerful influence over social mindset, and particularly children's concepts of themselves. For this reason, it is important that consumers understand exactly what ideas are being conveyed through these advertisements (Hein & Kahlenberg, 2009).

It is imperative that society be made cognizant of the intended and unintended messages presented through television advertisements, in order to help thwart adolescent viewers from accepting advertisers' gender biases. Evidence of persistent use of gender stereotypes supports the necessity for parents and educators to develop children's skills to critically analyze mediated messages. The following review of literature presents an overview of antecedent research pertaining to the use of stereotypical gender roles in commercials. This overview includes: (i) The use of stereotyping as a technique in advertising; (ii) Stereotypical messaging in children's commercials, and (iii) The use of gender-specific behaviors in children-targeted commercials.

The use of stereotyping as a technique in advertising: To understand the reasons that stereotypes are often used in advertising, it is necessary to examine the role that stereotypes can play in communication generally and message development in marketing communication, specifically advertising. The following review of the stereotype literature synthesizes two specific areas: the benefits and detriments of using stereotypes in advertising.

The Oxford English Dictionary (2015) defines a stereotype as "A preconceived and oversimplified idea of the characteristics which typify a person, situation, etc.; an attitude based on such a preconception". In other words, stereotypes are assumptions that ascribe certain characteristics to an overarching group of people, things or situations. Although the use of stereotypes generally has a negative connotation, in order to produce practical advertising, shortcuts are often used to deliver the advertiser's message in thirty seconds. There is a necessity for advertisers to craft commercials using symbolic meanings that align with cultural norms within a given society. For example,

culture frequently perceives symbolic meaning even from an element as arbitrary as color, such as red for love or anger and green for nature or jealousy. By strategically utilizing the predispositions existent within society to develop messages to promote ideas or sell products, advertisers can create more effective and even compelling advertisements that resonate with consumers' cultural values (McCracken, 1986).

Ifezue (2010) suggests that men and women process information in two distinct ways, indicating that these differences can play a significant role in the way that advertisers tailor messages to persuade each gender. For instance, men prefer to focus on the big picture, while women can process multiple pieces of information at once and generally have an eye for detail. With this information, it may be more effective for advertisers to layer a female-targeted advertisement with multiple components and more detail, while narrowing the focus of a male advertisement to one or two focal points.

Research suggests that in instances where women are positively portrayed from the women's perspective, advertising can be effective in selling a product without the use of negative stereotypes. Castillo, for example, found that 52 percent of women reported that they bought a product because they liked the way that advertisers presented women in the commercial (Castillo, 2014). Recent pro-female campaigns by brands such as Unilever and Always have been extremely successful in advertising (Mitchell, 2015). Dove's 'Real Beauty Sketches' campaign provide "a potent reminder to women that they are more beautiful than they think" (Nudd, 2013) and the #LikeAGirl campaign, an effort to reverse the negative connotation of the phrase "like a girl" (Mitchell, 2015), allows advertisers to respond to women's critique of the industry's use of negative stereotypes of women and girls.

Nevertheless, the positive approach is far from the norm. The use of outmoded symbolic meanings or traditional stereotypes in advertising is still prevalent, even though they can be ineffective and offensive, especially to women. While in some instances the use of stereotypical associations can increase the efficacy of advertising, in the case of gender stereotypes, women today respond more favorably to advertisements that portray them in less limited, more progressive roles. For example, Eisend, Plagemann, and Sollwedel (2014) found that women were more often aware of stereotypes in commercials than men.

In addition, research shows that women are more aware than men of the negative effects that stereotypes can have on society; thus, women are more likely to view commercials displaying traditional gender stereotypes negatively. For Eisend, Plagemann and Sollwedel (2014), a traditional stereotype denotes a woman in a domestic role, such as a woman cleaning while a man relaxes; in contrast, the nontraditional counterpart to this stereotype would feature a man cleaning while a woman relaxes. The results of the study indicate that female-targeted advertisements portraying women in nontraditional roles were more effective than those portraying traditional stereotypes (Eisend, Plagemann, & Sollwedel, 2014).

Stereotypical messaging in children's commercials: The examination of child-targeted commercials indicates that the use of stereotypical symbols in advertisements, such as traditional patterns in the gender of models, the pairing of certain products with only one particular gender, the gender of voice-overs and the gender association of setting, can all convey messages that may potentially restrict children's aspirations and self-realization.

Kolbe and Muehling (1995) observed that the models within a commercial impact children's perception of gender-appropriateness for the product being advertised. Their study found that 94.6 percent of children ages 5 to 9 could correctly identify the gender of a child playing with the toy in commercials. The gender of the model in the toy advertisement affected children's judgments about gender-appropriateness for that toy. Of the 40 participants in the study, 78 percent of the boys who saw a male actor playing with the toy felt that the toy was meant for "boys-only". However, boys who saw a female actress playing with the toy were twice as likely to feel that the toy was appropriate for girls and boys (67.4 percent). Girls were also less likely to view a toy as appropriate for "boys-only" if the advertisement contained the presence of a female actress.

Similarly, Hein and Kahlenberg (2009) suggest that advertisers teach children to embrace gender stereotypes by targeting a specific gender for different types of toys. Stereotypical "boy toys" were most often modeled by male actors and "girl toys" by female actresses, implying that certain toys are only appropriate for one sex or the other. For example, dolls (58.3 percent) and animals (82.6 percent) were more often featured in girls-only commercials, while

transportation/construction toys (87.1 percent), action figures (72 percent) and sports (63 percent) were all shown most often in boys-only commercials.

Additionally, the gender of voice-overs in commercials also appears to be selected based on the type of toy, which can indicate to children the gender-appropriateness of the toy. Martínez, Nicolás, and Salas (2013) found there was a male voiceover in 79.8 percent of commercials advertising a vehicle and a female voiceover in 66.1 percent of advertisements for dolls and accessories. Anuradha (2012) conducted a similar study researching the commercials aired in India, finding that no commercials targeted at boys utilized female voiceovers, but 39 percent of commercials targeted at girls used male voiceovers. Similarly, Centeno and Prieler (2013) showed that in the Philippine's advertisements, males dominated the area of voiceovers. This may suggest that either the male voice is considered to be more persuasive or fewer women are employed in the creation of commercials (Centeno & Prieler, 2013).

Finally, there appears to be a relationship between gender and the settings used in commercials. Similar to the traditional expectations that place women in domestic roles, children's advertisements tend to portray girls indoors more than outdoors (Anuradha, 2012). In their study, Hein and Kahlenberg (2010) found that about 84 percent of girls-only commercials featured girls playing inside, while 77.8 percent of the characters in commercials with an outdoor setting were boys. Along the same lines, boys are also shown in a work setting more often than girls (Davis, 2011; Hein & Kahlenberg, 2009).

The use of gender-specific behavior in child-targeted commercials: Researchers have also uncovered a multiplicity of findings regarding gender stereotypes pertaining to behavior (Anuradha, 2012; Bakir, Blodgett, & Rose, 2008; Hein & Kahlenberg, 2009; Klinger, Hamilton, & Cantrell, 2001). For example, boys in commercials targeted at child audiences are more likely to be shown in active roles, such as running or playing sports, while girls have the tendency to be given more passive roles, such as sitting and playing with dolls (Davis, 2011). Likewise, while independence is a trait typically more often associated with boy characters, advertisements most often show girls in cooperative roles, such as playing with other children (Anuradha, 2012; Hein & Kahlenberg, 2009). Hein and Kahlenberg (2009) found that while girls

were most often portrayed in cooperative roles (71.2 percent), boys were shown in a more diverse range of interactions, one of these being competitiveness. Over half (58.3 percent) of the competition displayed in the commercials was depicted in commercials with only boys, while none of the girls-only commercials featured competitive behavior.

Furthermore, children appear to note differences in behaviors in commercials. An experimental study found that both boys and girls rated male-focused advertisements as more aggressive than female-focused commercials (Klinger, Hamilton, & Cantrell, 2001). However, girls rated imagined play with boys' toys as having a higher degree of aggression than boys rated them. The authors of the experiment concluded that media's repeated exposure of violent behavior may be socializing boys toward aggression, noting as well that this could lead eventually to boys' desensitization toward this behavior.

In this study, the author examines stereotypical messaging in children's commercials and also analyzes the use of gender-specific behaviors in prime-time commercials targeted to children. This work adds to antecedent research by examining the additional behaviors of athleticism and dancing. Athleticism, defined as natural physical skill, agility, strength or talent pertaining to sports, can sometimes be a manifestation of dominance, similar to competitiveness and is, thus, a behavior expected to be associated more with boys. Contrastingly, because girls are often portrayed as more docile, playful and cheerful, perhaps dancing may be an athletic behavior that is more associated with girls in commercials (Bakir, Blodgett, & Rose, 2008; Klinger, Hamilton, & Cantrell, 2001).

Methodology

This study used content analysis to examine a one-week composite sample of 200 television commercials broadcasted on Nickelodeon network in July, 2014, from 3:00 to 7:00 p.m. on weekdays and 10 a.m. to 2 p.m. on Saturday and Sunday. In order to collect a comprehensive representative sample, the study selected a day-part during weekday after-school hours and a morning day-part on the weekend. These time frames were selected to correspond with the day-parts in which children view the most television (Morgan, 2013; Trefis team, 2014). Nickelodeon was selected as the focus for this study because it was named basic

able's number-one network for the second quarter of 2014, with 834,000 total day viewers ages 2-11 and 1.6 million total viewers (Viacom, 2014).

The unit of analysis for this study was each commercial shown during the two time frames. Duplicate commercials were included in the content analysis since the literature suggests that the more a child views a commercial, the more likely it is that the child's perception of reality will be influenced by its content (Kahlenberg & Hein, 2009).

In order to analyze the content of current children-targeted commercials and to determine to what extent gender stereotypes are being used, a content analysis was considered the most appropriate methodology for this study. Manifest information observed in the commercials was coded independently by three coders. After the author trained the coders, each coder independently coded 5% of the total commercials to establish appropriate inter-coder reliability. Using Holsti's method (Poindexter & McCombs, 2000), inter-coder reliability was calculated to be 98 percent agreement. However, in order to account for any amount of inter-coder agreement that could have occurred by chance, the Scott's pi method of inter-coder reliability was also calculated separately for variable 18 of a randomly selected commercial. For this variable, Scott's pi was calculated to be 97 percent agreement. This variable (V18) was selected for this additional calculation because it asks the coder to select the *most* dominant behavior displayed by the main character, thus subjecting the coder to a higher degree of personal judgment, in comparison to other variables.

In order to determine what messages commercials are relaying to children about gender roles today, this content analysis tested 6 hypotheses. They are as follows:

- H1. Commercials with action figures and construction, building or transportation toys will feature only male actors
- H2. Commercials with dolls and stuffed animals will feature only female actresses
- H3. More boys-only commercials will feature boys in competition
- H4. More girls-only commercials will feature girls in a nurturing role
- H5. More boys-only commercials will feature aggression or fighting than girls-only commercials
- H6. More girls-only commercials than boys-only commercials will feature girls playing indoors

A coding scheme was developed to facilitate the objective description of manifest content in order to make valid inferences about (i) Gender Appropriateness Depicted in Toy Commercials and (ii) Gender Specific Characteristics in Commercials.

Gender Appropriateness Depicted in Toy Commercials: To determine the relationship between the toy advertised and the gender of the children being targeted, type of product advertised and the gender composition of the characters or actors were analyzed in the commercial (Hein & Kahlenberg, 2009; Kolbe & Muehling, 1995).

To determine whether there were certain types of products that were associated with girls and another set associated with boys, advertised products in the commercials targeted to children were coded as *type of product* being advertised, grouping the coding categories of products as "toy", "food/beverage", "make-up/beauty product", "clothing", or "arts and crafts".

Types of toys were classified as "action figures", "construction/building oriented", "transportation", "dolls", "stuffed animals", "sports/outdoor", "technology related", or "board games" (Hein & Kahlenberg, 2009). With regard to the specific toys mentioned in Hypotheses 1 and 2, this study replicates that of Hein and Kahlenberg (2009) whereby action figures, construction, building or transportation toys were classified as "boy toys", and dolls and stuffed animals were classified as "girl toys".

Gender Specific Characteristics in Commercials: In order to capture the overall proportion of gender represented by the characters on screen, *Gender of characters* was coded as "girls-only", "boys-only", "more boys than girls", "more girls than boys", "equal amount of boys and girls" or "ambiguous". Since the gender of the narrator of a commercial is often distinguishable and may impact an audience's perception of a commercial, the *Gender of narrator* was coded as "male adult", "male child", "female adult", "female child", "no narrator", "ambiguous", or "unsure".

The following definitions provide insight into the meaning of the terms used to describe behaviors and interactions analyzed in this study.

Behavior displayed by characters

1. **Competitive:** characters showing a strong desire to win a game/competition (i.e. sport, competition with goal to win)
2. **Nurturing:** characters shown coddling, feeding, nursing or taking care of a doll, animal or another character
3. **Aggressive:** characters or toys shown battling, wrestling or using physical force (fighting for sake of fighting)
4. **Athleticism:** characters shown playing or using some form of physical talent/skill, but not competing with main goal to win (i.e. hiking, swimming but not racing, running etc.)
5. **Dancing:** moving along in the rhythm of music

Interactive behavior

1. **Cooperative:** two or more characters playing and interacting together
2. **Parallel:** two or more characters shown together, but not playing/interacting directly with one another
3. **Independent:** only one child was shown playing in the commercial

For the purposes of better understanding the behavioral stereotypes currently associated with gender and to draw a comparison of stereotypes that have been most prominently discussed in the literature, *behavior displayed by characters* was coded as “competitive”, “nurturing”, “aggressive”, “athletic”, or “dancing”. *Interactive behavior of characters* was coded as “cooperative”, “parallel”, or “independent”.

To determine if there was any difference in the environment that was associated with commercials with girls or boys, the environment or location was analyzed. *Location* was coded as “indoors” or “outdoors”.

Race of the characters was coded to analyze the observed races represented in the commercials. The options for this variable included “Caucasian”, “African American”, “Asian”, “Hispanic” and various combinations of those races.

Data analyses were conducted with the use of SPSS software. Cross tabulations were performed with *Gender of Characters* and one other pertinent variable and Pearson’s chi-square tests were used to determine whether there was significance between the expected

frequencies and observed in all the coding categories.

Results

Description of the Advertised Products

From the sample of 200 commercials analyzed, two-thirds of the commercials were for food /beverages, movies, toys or clothing. Thirty-seven percent of the commercials advertised food or beverages, 11 percent were movies, 10.5 percent were toys, 6.5 percent were clothing items, 5 percent were insurance companies, 4 percent were arts and crafts, 4 percent were online teaching tools and 19.5 percent fell under “other” which included, Chuck E. Cheese (2.5 percent), Febreze air freshener (2 percent), Pampers diapers (1.5 percent), babysitting websites (1.5 percent), and others.

Cereal commercials constituted half (50 percent) of all the food products advertised, followed by snack foods (20 percent) and fast food (10.5 percent). In addition, 8 percent of food commercials advertised candy or gum and 6.5 percent featured juices.

Dolls represented the largest percentage of toys advertised (32 percent), followed by technology-related toys such as computer games or educational websites (23 percent) and construction/building toys (18 percent). Shoes represented 82 percent of the clothing items advertised. Most often, the shoes advertised were athletic-type shoes or sneakers (primarily Sketchers).

Description of Setting

Less than half (44 percent) of the commercials were set only indoors, 23 percent were set only outdoors, and 28.5 percent featured scenes that were both indoors and outdoors. None of the commercials with only girl characters were set outdoors, while 63.6 percent of girls-only commercials were set indoors. Similarly, commercials with more girls than boys were more frequently set indoors (41.7 percent) than outdoors (16.7 percent). However, the difference between the percent of boys-only commercials set indoors (31.4 percent) and those set outdoors (37.1 percent) is much smaller. Similar results were found for commercials with more boys than girls. Please refer to the figure below for a depiction of the findings.

Gender Relationship with Setting

Gender Representation in Commercials	Indoor	Outdoor	Indoor & Outdoor
Girls-only	63.6% [7]	0.0% [0]	36.4% [4]
Equal boys and girls	60.8% [31]	13.7% [7]	25.5% [13]
More girls than boys	41.7% [10]	16.7% [4]	41.7% [10]
More boys than girls	34.6% [18]	30.8% [16]	34.6% [18]
Boys-only	31.4% [11]	37.1% [13]	31.4% [11]

Figure 1 depicts the statistically significant relationship between the gender of characters in children's commercials and the setting that those characters are portrayed in. While male-dominant commercials are likely to be shown indoors or outdoors, female-dominant commercials are significantly more likely to be shown only indoors.

Description of Characters

Overall, 30 percent of commercials featured only one main character, 24 percent featured two main characters, 17.5 percent featured three main characters, 18 percent contained four main characters and 10 percent had no obvious "main" characters. More than half (52 percent) of the characters in the study were real people, 19 percent were cartoon or animated animals, 11.5 percent were cartoon people, 10 percent were a combination of real people and animated characters and 7.5 percent were some other type of character representation, such as imaginary creatures or animated inanimate objects.

Of all the commercials analyzed, the majority (62.3 percent) had only Caucasian characters, followed by African American (9.6 percent) and Asian (1.8 percent) characters. For commercials where only one race was represented, the finding was similar.

Overall Gender Representation of Characters and Narrators

The gender representation of the commercials is exhibited in figure 5. Boys were represented by themselves in the commercials three times more than girls. Only 6.3 percent [11] of commercials had only girls, while 21 percent [37] of commercials featured only boys and almost 30 percent of commercials featured more boys than girls. About thirty percent featured equal amounts of boys and girls, while only

13.6 percent featured more girls than boys.

Over three-quarters of the commercials had narrators and men dominated in that role. Nearly 60 percent of the narration featured in commercials was of male adult voices. Twenty-one percent featured a female adult narrator, while 11.5 percent had no narrator. Children narrated only 7.5 percent of the commercials, most of which (4.5 percent) featured a male child.

Gender Relationship with Type of Toy

The results of the study support Hypotheses 1 and 2, which predicted that action figures and construction, building or transportation toys would feature only male actors, while doll and stuffed animal commercials would feature mainly female actresses.

There was a statistically significant relationship (*chi-square* = .005) between the gender representation and type of toys. For example, commercials advertising traditional "boy toys" had only boys or primarily boys featured in the commercial and traditional "girl toys" advertisements only featured girl characters in the commercials.

None of the "boy toys" advertisements featured either "girls-only" or "more girls than boys" in the commercials. All of the commercials that advertised action figures, construction/building or transportation toys featured boys-only (33.3 percent) or more boys than girls (66.7 percent) characters or models. Likewise, none of the commercials advertising dolls or stuffed animals featured "boys-only" or "more boys than girls". The majority of "girl toy" advertisements featured "more girls than boys" (60 percent), and the rest featured only girls (40 percent). None of the commercials advertising either "boy toys" or "girl toys" featured equal amounts of boys and girls.

Sixty percent of technology-related toys were advertised in boys-only commercials. Although 20 percent of these toys were advertised in commercials with more girls than boys; 20 percent were also in commercials with equal numbers of boys and girls.

There was a distinct pattern observed in how toys were advertised in commercials targeted to children. Within boys-only commercials, 100 percent of toys advertised were "boys' toys", meaning action figures, construction/building toys and transportation toys. Likewise within the girls-only commercials, 100 percent of toys advertised were dolls or stuffed animals.

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Description of Characters’ Behavior and Interaction

The most frequent behavior featured in the commercials was aggression, followed by dancing, competitiveness, nurturing and athleticism. With 72 main characters displaying aggression as their most dominant behavior, this represented over one quarter (27 percent) of the commercials. Following close behind were the behaviors of dancing (23.6 percent each) and competitiveness (22.9 percent). Nurturing (18.1 percent) and athleticism (8.9 percent) were the behaviors least observed in the commercials. Figure 2 depicts the overall representation of these behaviors.

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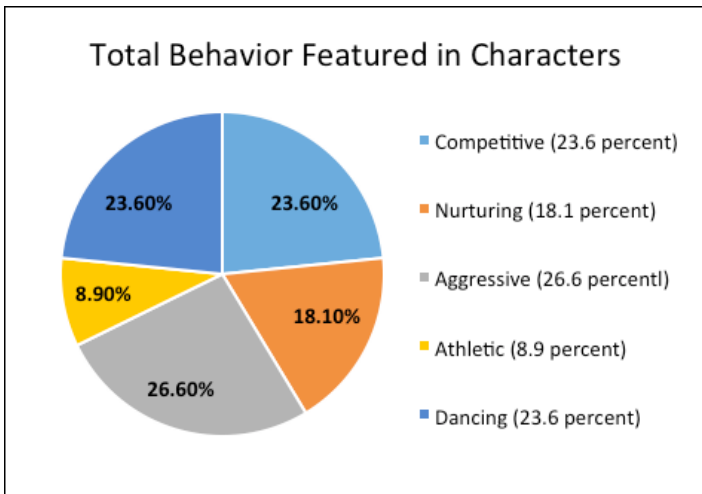


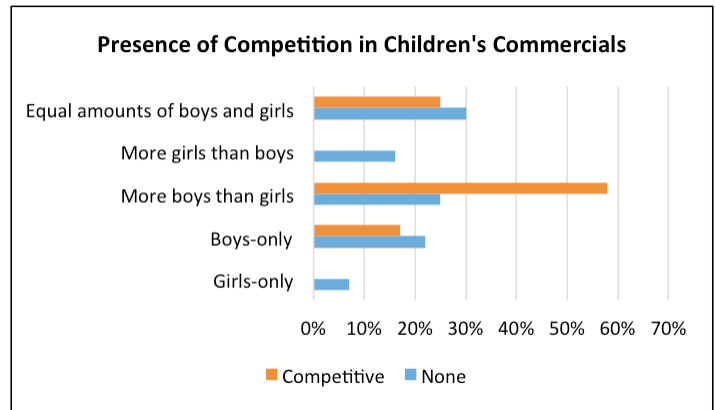
Figure 2 illustrates the representation of five traditionally gender-specific behaviors in children’s commercials

each) and competitiveness (22.9 percent). Nurturing (18.1 percent) and athleticism (8.9 percent) were the behaviors least observed in the commercials. Figure 2 depicts the overall representation of these behaviors. Overall, independent behavior (47.9 percent) was the most common behavior displayed in children’s commercials. The next most frequently portrayed behavior was cooperation (37.5 percent). Only 8.3 percent of commercials depicted parallel behavior.

The Relationship between Gender and Type of Behavior

Chi-square analyses reveal a statistically significant relationship between gender and behaviors such as competition, nurturing and aggression, which are traditionally used in a stereotypical manner in commercials targeted to children. However, results also indicate that athletic behavior and dancing show no statistically significant relationship to gender.

Results indicate a statistically significant relationship between gender and competition ($p=.008$). The highest percentage of competitive behavior was featured in commercials that had more boy than girl (58 percent) characters. Twenty-five percent of commercials that displayed competition were advertisements with equal amounts of boys and girls, while 17 percent of competitiveness was displayed in commercials with only boys. Neither girls-only commercials nor commercials with more girls than boys displayed any instance of competition. Please refer to Figure 3 for more information on these findings.



($X^2 = 13.766^a$, 4df, $p=.008$) N=200

Figure 3 shows the statistically significant relationship between competitive behavior and gender. Boys are significantly more likely to be portrayed in competitive roles than girls.

Figure 4 shows that nurturing behavior was found to be most prevalent in commercials with equal amounts of boys and girls (38 percent) and in commercials with more girls than boys (35 percent). Fifteen percent of nurturing behavior was present in girls-only commercials, while four percent was accounted for in boys-only commercials. Commercials with more boys than girls displayed only eight percent of the total nurturing behavior, while those with more girls than boys displayed 35 percent of the total nurturing behavior. These results indicate that there is a statistically significant relationship ($p= .000$) between female-targeted commercials and nurturing behavior.

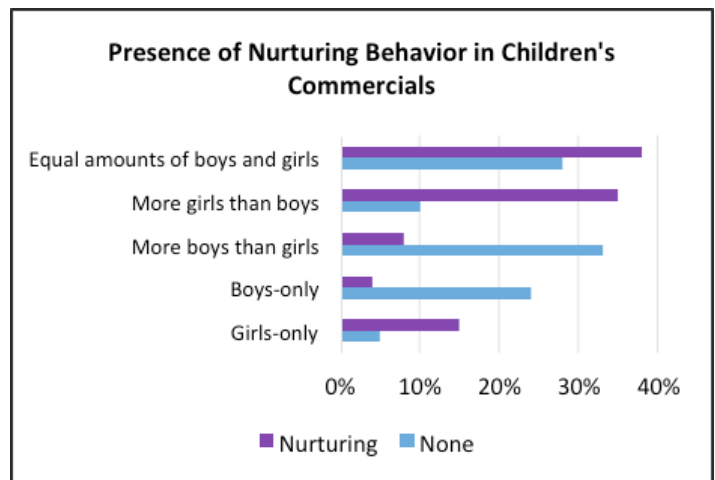
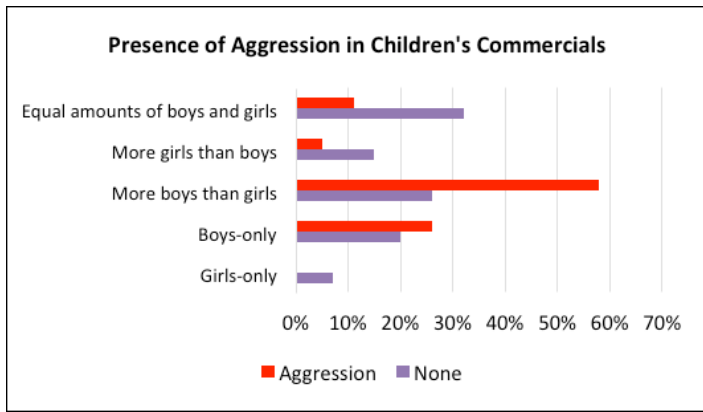


Figure 4 illustrates the statistically significant tendency of children’s advertisements to portray nurturing behavior in female-dominant commercials.



*($\chi^2 = 11.124^a$, 4df, $p = .025$) N=200

Figure 5 depicts the statistically significant relationship between male-dominant advertisements and aggressive behavior.

There is a statistically significant relationship between aggression and gender ($p = .025$). Aggression, the most frequently occurring of the behaviors observed in commercials, was found more in commercials with boys than girls (58 percent) and boys-only (26 percent). There were no “girls-only” commercials that displayed aggressive behavior and only five percent of those with more girls than boys demonstrated any aggressive behavior. Please refer to Figure 5 for more detailed information.

There was no statistically significant correlation found between gender and athleticism in children’s commercials. Athletic behavior was found to be most prevalent in boys-only commercials and commercials with equal amounts of boys and girls (31 percent each). The next highest proportion of commercials with athleticism consisted of those with more boys than girls, at 23 percent.

Results indicate that dancing was more commonly observed in boys-only than girls-only commercials but, like athletics, there was no statistically significant association between gender and dancing found in the commercials. Findings suggest that dancing was most common in commercials with an equal representation of both genders (28 percent) and in commercials with more boys than girls (28 percent). However, the next highest proportion of dancing took place in commercials with more girls than boys, at 24 percent. Fourteen percent of the boys-only commercials featured characters dancing, while seven percent of girls-only commercials portrayed dancing.

Discussion

Many longstanding stereotypes still remain in commercials targeted to children, although there is evidence that advertising has taken some steps toward a more nontraditional representation of gender. The results of a systematic content analysis of children’s commercials aired on the Nickelodeon network during prime viewing times reveal that while not all gender roles portrayed in advertising targeted to children have remained stagnant, many of the gender stereotypes from the past three decades continue to endure today.

Mirroring the same pattern from years of antecedent research, this study found a strong correlation between gender in children’s commercials and the toy being advertised. This correlation results in the perpetuation of the stereotype that certain toys are for boys and other toys are for girls. The analysis found a statistically significant relationship between gender and types of toys ($p = .005$). Similar to the findings of Hein and Kahlenberg (2009), the results of this study supported Hypotheses 1 and 2, which predicted that toy products such as action figures and construction/building or transportation toys would generally feature male actors, while dolls and stuffed animals would be advertised using predominantly female actresses. Within the commercials that only featured girl characters, 100 percent of the commercials were advertising a “girl toy”, a doll or a stuffed animal, while 100 percent of boys-only commercials advertised a “boy toy”, such as a toy truck or a set of Legos. Additionally, technology toys were targeted primarily to boys. Three-fifths of technology-related toys, such as video or computer games, featured only boy characters.

Overall, results reveal the enduring existence of a male-dominant representation of gender in children’s advertisements. The data clearly show that boys are pictured 70 percent more frequently in children’s commercials than are girls, even though boys age 2 to 9 years old only make up approximately one percent more of the population than girls of the same age (US Census, 2012). The strong prevalence of male-dominated advertisements, including both exclusively-male advertisements and commercials with more boys than girls, suggests that advertisers are continuing to give male children more opportunities to be visible and vocal in mediated spaces.

The narrators of the commercials in the study tend to be primarily male (64.3 percent), further

emphasizing the dominant presence of boys. A narrator in a commercial is the voice of information and persuasion, two noteworthy sources of social power. The prevalence of male narrators serves as supplementary evidence to the view that advertising reinforces not only male-dominated commercials, but also a male-dominated society.

For decades, advertising research has indicated that women are most often depicted in the home, while men are shown at work or outdoors (Eisend & Knoll, 2011; Hein & Kahlenberg, 2009). In support of this literature, this study found a significant relationship between gender and setting ($p = .028$). For example, while 63.6 percent of “girls-only” commercials were indoors, only 31.4 percent of “boys-only” commercials were indoors. Furthermore, no girls-only commercials were set outdoors. Overall, these findings add evidence to the view that women are or should be more domesticated, while boys are more “outdoorsy”. By showing girls mainly indoors, these commercials perpetuate the narrative that a woman’s place is in the home, while men have the option of exploring a wider range of environments. The implication is that girls and women are expected to remain within the boundaries of domestic environments and men are free of such limitations.

In addition to the product association and the traditional gender-oriented settings, stereotypical behaviors such as aggression and competition also continue to permeate boy-targeted children’s commercials, while perceived unassuming behavior, such as nurturing, tends to be most associated with girls.

Advertisers showed competitive behavior in commercials where boys were the main actors: Competitiveness was noticeably absent from commercials with primarily girls. Anuradha (2012) and Hein and Kahlenberg (2009) found that children’s advertisements tend to characterize boys as more competitive than girls. The results of this study mirror these findings and support Hypothesis 3, as boys were found to be statistically significantly ($p = .008$) more likely than girls to show competitive behavior in the commercials. No girls-only commercials showed competitive behavior, but 16.7 percent of competition was shown in boys-only commercials. Furthermore, no competition was present in commercials with more girls than boys; nonetheless 58 percent of all competitive behavior was displayed in commercials with more boys than girls. The implication of this finding is that commercials are socializing girls

to avoid competition.

However, 25 percent of commercials with “equal amounts of boys and girls” exhibited competitive behavior, similar in comparison with the 22 percent in Hein and Kahlenberg’s (2009) findings. While this statistic appears to reflect a step away from the traditional stereotype that competition is a realm limited to boys only, it also seems to imply that girls compete more when boys are present, as there were no girls-only commercials that featured competitive behavior.

Nurturing behavior appears to permeate girl-dominant commercials: Nurturing behavior is evident in commercials with equal amount of boys and girls and in girls-only commercials, but still noticeably absent in boy-dominant commercials. Domesticity has traditionally been a trait associated with women (Bakir, Blodgett, & Rose, 2008; Davis, 2011; Eisend & Knoll, 2011). In children, one manifestation of this stereotype can be seen through nurturing behavior, such as caring for, feeding or cuddling a doll or stuffed animal. Hypothesis 4 was supported in that nurturing behavior tends to be more prevalent in girls, as 36.4 percent of “girls-only” commercials displayed nurturing behavior, while only 2.7 percent of boys-only commercials showed nurturing behavior. This was a statistically significant relationship ($p = .000$). Results also offered evidence that advertisers seem to be making some advances away from stereotypical gender roles, as the largest percentage of commercials displaying nurturing behavior were those featuring “equal amounts of boys and girls” (38.5 percent). Nonetheless, the deficiency of nurturing behavior in boys-only commercials continues to support the expectation that nurturing behavior is a tendency primarily ascribed to girls. The implication of this finding is that boys are rarely being exposed to images in commercials of males being nurturers without the presence of girls. Boys perhaps are being socialized that being nurturing is not a role for boys and men.

Aggression was found to be more prevalent in male-dominant children commercials: In regard to Hypothesis 5, which predicted that boys would be portrayed as more aggressive than girls, this study’s findings were similar to that of Klinger, Hamilton and Cantrell (2001) and Browne (1998), in that boys are portrayed to be more aggressive than girls in children’s advertisements. Most commercials displaying aggression had more boys than girls (57.9 percent), or had only boys (26.3 percent). In contrast, there were no “girls-only” commercials that displayed any

aggressive behavior. Across the board, these results reflect longstanding social expectations that women and girls should not demonstrate aggressive behaviors, yet this is an expectation of men and boys. While negative aggression is not condoned by this author, the implication of this finding is that it is acceptable for boys to fight, wrestle or use physical force, while this is not an expectation for girls. The study did not make observations as to which gender was the recipient of the male-dominated aggression.

A propensity to show athleticism in boys was observed in this study, but athletic behaviors were also evident among girls: Although traditional gender expectations would posit that boys should be characterized as more athletic than girls, advertisements aired on Nickelodeon seem to be making strides toward a more progressive, non-traditional representation of girls. There was not a statistically significant relationship ($p=.328$) found between gender and athleticism. Although there was a clear propensity for athletic behavior in boys-only commercials (31 percent) as opposed to the 15 percent of athleticism portrayed in girls-only commercials, there was an equal display of athleticism in commercials with both boys and girls (31 percent).

Dancing appears to be a gender-neutral behavior: Given that there exists a social expectation for boys to be masculine, independent and professional, while girls are viewed as cooperative, less serious and more animated, it would follow that girls would be more associated with dancing (Anuradha, 2012; Hein & Kahlenberg, 2009; Neto & Pinto, 1998). However, the results of this content analysis would seem to contradict this expectation. With a p -value of .426, dancing and gender were not shown to have a statistically significant relationship. Overall, commercials targeted to children do not appear to strongly link dancing to either gender. The highest proportion of commercials featuring characters dancing were advertisements with equal amounts of boys and girls (28 percent) and commercials with more boys than girls (28 percent). A possible explanation for this finding is that dancing is an activity that is often done with a male and female together. That said, the study found that there were twice as many boys-only commercials (14 percent) displaying characters dancing in comparison to girls-only commercials (7 percent).

In summary, hypotheses 1 and 2 were supported, in that a statistically significant relationship was found between boys and “boy toys” and, conversely, girls and

“girl toys”. Furthermore, hypotheses 3 through 6 were also supported by results in that there was a statistically significant association between boys and the behaviors of competition and aggression, as well as girls and nurturing behavior and a domestic setting.

Conclusion

In conclusion, this content analysis contributes to antecedent advertising research in the area of gender stereotyping. Results indicate the clear presence of longstanding gender expectations within current children’s commercials, including the association of toy types, setting, nurturing, competitive and aggressive behaviors with a particular gender. However, findings also demonstrate nontraditional strides within advertising toward a more contemporary notion of gender, particularly in the areas of athleticism and dancing. The progress away from gender prejudice in the area of athleticism coincides with recent Femvertising campaigns, such as the #LikeAGirl campaign, which encourages women to proudly run and throw ‘like a girl’ (Mitchell, 2015). Still, there remains ample room for progress to be made.

While stereotypes will perhaps always remain a component of advertising, if progress continues, the gender stereotypes that saturate commercials today may one day cease to ensnare children’s perception of themselves. For advertising to be more effective, it needs to keep abreast with cultural and attitudinal changes in women and girls.

Although this study certainly provided support for previous research that indicated a statistically significant association between gender and the types of toys advertised and certain behaviors, a broader sample size would have provided a clearer understanding of these relationships. Analyses of 200 commercials did not allow for an all-encompassing view of certain types of behavioral concepts depicted in the commercials.

Future studies can further the research of gender stereotypes in children’s advertising by analyzing a broader range of children’s networks. Studies could also analyze parents’ attitudes and perceptions of stereotypes depicted in children’s commercials, as well as commercials directed at adults on children’s networks. This study found that commercials for products such as insurance, Pampers diapers and baby-sitting services were clearly targeted to parents. It is possible that the degree of gender stereotyping and even the type of stereotyping used may differ between children-targeted

commercials and adult-targeted commercials. Finally, with the increasing popularity of electronic games, this research could be further advanced by taking a more concentrated focus on the gender stereotypes associated with video games.

Overall, this study reveals that gender stereotypes remain prevalent within contemporary children's advertisements. Thus, advertisers will likely continue to market Pocket Polly to girls who are exhibiting different behaviors from boys who prefer to play with Ninja Turtles. However, the results of the study also indicate that some of the traditional stereotypes that have characterized children's commercials for the past few decades are now showing signs of transformation.

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**Mortal Reason and Divine Infinity:
Justifying the Ways of God to Men in Book VI of *Paradise Lost***

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Abstract

In his epic poem, *Paradise Lost*, Milton's goal was to "justify the ways of God to men" (*PL* I.25-26). For his seventeenth-century Protestant audience, this meant reconciling both the paradox of human free will and divine foreknowledge and the paradox of human suffering and God's goodness. Although God's speech in Book III makes an explicit argument declaring God's justice, this paper will show that Book VI, the War in Heaven, completes this argument by attempting to move the poem's readers beyond the limits of human reason into a divine understanding of the universe. Through temporal compression and confusion, created by the language of Book VI and Satan's creation of the cannon, the poem elevates the reader from mortal temporality to divine infinity. This perspective, which approximates God's omniscience, just as the War in Heaven simulates human suffering, allows post-lapsarian humans to understand intuitively how those paradoxes may be harmoniously resolved.

[I]nto hollow engines long and round
Think-rammed, at th'other bore with touch of fire
Dilated and infuriate [they] shall send forth
From far with thund'ring noise among our foes
Such implements of mischief as shall dash
To pienes and o'erwhelm whatever stands
Adverse...

(PL VI. 484-490)

So Satan commands the construction of the most discordant weapon of the War in Heaven in Book VI of *Paradise Lost*: the cannon, which was a state-of-the-art weapon of mass destruction for Milton's contemporaries. In Raphael's account, the heavenly angels are indeed dashed about and nearly overwhelmed by the devilish creation, but Milton's reader is also unsettled by the cannon, having been immersed just moments before in a poem written in the style of a Homeric epic and set in the Garden of Eden.

Even more jarring than Raphael's description of Satan's terrifying invention is the aside that follows shortly afterward. Raphael switches mid-line from narrating the admiring thoughts of Satan's followers to addressing Adam directly. He warns him that "if malice should abound," that is, if mankind should fall into sin, it is likely that some future human will develop an earthly form of the cannon "to plague the sons of man" (*PL VI.501-506*). Raphael then returns to his narrative

without missing a beat. This comment is baffling. It jolts the reader out of the flow of Raphael's story and draws attention to the fact that he is relating events where the outcome—Satan's defeat—has already been foreshown in the poem, reducing the tension of the conflict.

While there are other direct addresses to Adam in Book VI, one near the beginning and one near the end, this one stands alone both in placement and in the degree to which it breaks the flow of the epic narrative.

¹ Raphael's reference to Adam as his listener in the last lines of the book coincides with the end of the story of the War in Heaven. His other switch into the second person is near the beginning of the book, and comes as part of an epic simile comparing the ranks of marching angels to the birds that came flying to Adam in paradise "to receive / Their names of thee" (*PL VI.75-76*). Although this does detour from the narration to some extent, it is a much smoother interruption that more neatly integrates past and present, Heaven and Eden, through the medium of the simile. In contrast, after over

¹The narrator sometimes refers to himself in the first person or makes a reference to the fact that he is telling a story—for example, when he remarks that darkness in Heaven is like "twilight here" (*PL VI.12*). However, these moments are not as jarring as those in which we are suddenly reminded that for hundreds of lines, we have been hearing Raphael's narration rather than the general narrative voice chosen by Milton to represent himself more directly.

four hundred lines of uninterrupted epic presentation that draws us into an illusionary intimacy and erases Raphael and Adam, the transition from narration to direct address during the construction of the cannon is disorienting in its suddenness, to the point that it seems almost sloppy.

There are some obvious explanations for this brief passage about post-lapsarian human war, just as there are some clear and unsubtle reasons for the inclusion of the cannon. The cannon could be a reflection by Milton on the horrors of seventeenth-century warfare, or perhaps he simply wanted to give his great villain access to the most terrifying weapon of his time. Likewise, Raphael's aside could have been included merely to point out to Milton's readers that the horrors of modern warfare are a result of human sin, or, within the narrative, to be a specific warning for Adam about the dangers posed by Satan.

Yet the placement of the passage makes little sense if these were the main reasons for its inclusion. The idea that the sufferings of war are the result of the fall would be such a familiar and well-accepted concept to Milton's readers that it is difficult to imagine why he would willingly interrupt the rhythm of the epic narrative to point out the obvious. As an intratextual warning to Adam, it is equally surprisingly placed because at this point Satan has only introduced the idea for the cannon. While his description of its purpose is certainly alarming, Adam has yet to hear about its actual destructive impact. Raphael's prediction would be much more effective later on in the book, after he relates how the cannon's "roar / Emboweled with outrageous noise the air" as "her entrails tore disgorging foul / Their dev'lish glut: chained thunderbolts and hail / Of iron globes" and the angels "fell / By thousands" (*PL* VI.586-587, VI.588-590, VI.593-594). As it is, Milton placed the passage to be as disorienting to the reader as possible.

By reminding the readers that we are with Adam, listening to a story about the past, Milton creates a moment in his poem where his readers are simultaneously aware of and experiencing the past (the War in Heaven), the present (Raphael and Adam's conversation), and the future (Milton's time, when cannons are in use). In other words, the effect of the passage is not just to warn Adam, but also to give the poem's readers the faintest taste of God's omniscience. We do not become omniscient, but for a moment the poem allows us to get as close as possible to the

experience of what it might be like to exist in multiple times simultaneously.

This elevation into a divinely omniscient perspective is not merely an interesting rhetorical trick. Rather, it is a significant facet of Milton's main argument in the poem. In the opening of *Paradise Lost*, Milton invokes his Muse, the Holy Spirit, to grant him "Eternal Providence" that he may achieve his goal for the epic: to "justify the ways of God to men" (*PL* I.25-26). Milton believed in a God that was infinite, eternal, omnipresent, omnipotent, and omniscient (Fallon 33). Yet he also believed that humanity fell of its own free will and that God both created the circumstances of, and foreknew, that fall. The paradoxes implicit in these beliefs—that God is all-powerful and all-knowing, yet humanity has free will; that humanity fell under the circumstances of temptation and divine foreknowledge of the fall, yet God is "just"—seem to surpass the abilities of human understanding.

The problem of divine justice, especially with regard to the fall, was being hotly debated during Milton's time (Gregory 178). Milton's Arminian beliefs regarding divine foreknowledge and mortal free will were in the minority in seventeenth-century England, especially in Puritan circles (Gregory 178). He rejected Calvinist predestination and believed absolutely in the freedom of will (Gregory 202-3). On top of this, he was also wrestling with the standard challenge of the Reformation theologian to present an all-powerful God that was not a tyrant, and who was wholly good despite the undeniable presence of evil in the world (Donnelly, *Milton's* 18, 78). Raphael tells Adam that the horrors of gunpowder warfare are a result of man falling into sin, but such anguish is difficult to reconcile with the idea of an omnipotent, omniscient, and benevolent and just Christian God.

With such difficult paradoxes to face, and sharing his beliefs with only a minority of his contemporaries, it is no wonder that, as Gregory points out, Milton felt a strong defense of God's justice was actually necessary (202). It was not, of course, necessary for God's sake—Milton is not putting God on trial, even to absolve him of wrongdoing. Even the frustrated, fallen Adam acknowledges that God needs no justifying in this sense (Reist 236). Instead, as Reist and many other scholars have recognized, Milton is justifying God for the sake of his contemporaries (238).

Milton's goal with *Paradise Lost* was to reconcile these paradoxes by telling the story of the fall,

the loss of Eden. For Milton, human understanding may not be enough to resolve these problems comfortably, but that is a failure of human understanding, not of God. Therefore, in addition to using more traditional, “discursive” kinds of reasoning and logic, he pursued this purpose by attempting to bring his readers closer to a timeless and infinite divine perspective through what Milton’s Raphael calls “intuitive” reasoning (*PL* V.488). Primarily through temporal manipulation within the narrative space of the poem, especially in Book VI, Milton sought to move his readers beyond the limits of human reason into an understanding of the universe and the Fall through approximate experience of God’s perspective, or at least experience closer than might normally be possible.

Milton’s views on reason provide a useful tool for interpreting the methods he employs to construct his “great argument” (*PL* I.24). In particular, an examination of what Milton meant by “intuitive” and “discursive” reveals why he would not have been satisfied with leaving his argument on behalf of God’s justice to explicit explanations, such as the one given by God in Book III. Furthermore, it demonstrates how an important facet of his attempt at justification would have been leading his readers to an implicit understanding through indirect poetic means, such as raising them closer to a divine perspective. Firmly establishing Milton’s beliefs about the relationship between human and divine reason is an essential prerequisite to understanding how these beliefs play out in the text of the poem.

[T]he soul
Reason receives, and reason is her being,
Discursive or intuitive: discourse
Is ofttest yours, the latter most is ours,
Differing but in degree, of kind the same.

(*PL* V.486-490)

So Raphael lectures the yet-unfallen Adam in the Garden of Eden. The angel simultaneously affirms that reason is an essential quality shared by human and divine beings, the “being” of the soul, while marking a clear distinction between its two degrees: lower human discourse and higher angelic intuition. These assertions, put into the mouth of an angel, are a window into Milton’s own beliefs about the degrees and uses of reason.

Milton’s distinction between intuitive and discursive reason stems from his Ramism and the idea

of “right reason.” Ramism, the logical system derived by Peter Ramus in the sixteenth century, rejected convoluted Aristotelian logic and focused instead on reason as the intuitive and natural understanding of the relationships between things (Duhamel 1036-37). While Milton disagreed with Ramus in several major respects and revised the Ramist method in his own *Artis Logicae*, he did agree with Ramus’s emphasis on intuitive perception.² His versions of reason and logic promoted the use of imagination and “cultivated understanding” over a reliance on intricate proofs (Fisher 38). He saw *a priori* axioms—that is, intuitively understood truths—to be superior to pure logic (Arnold 22). Logic is part of reason, but reason is much more, and of a much higher faculty, than just logic (Arnold ix). In the *Artis Logicae*, Milton distinguishes between the practice of “reasoning” and “dialectic,” the latter of which he regards as too limiting because it only refers to “the art of questioning and answering” (*Works* v.11, 19-21).

The concept of “right reason” is much older and much more well established than the ideas of Ramus. It has its origins in the Plato’s dialogues and was given its name by Cicero (Arnold 1). It is, most simply described, the “simultaneous act of right knowing *and* right doing”; right reason is, in a sense, righteous reason (Arnold 2). It is reason that approaches intuition through divinely granted inspired insight (Fisher 41). Christian church fathers adopted it and brought it into a Christian context. For example, Augustine’s *sapientia* is “the contemplation of the truth, tranquillizing the whole man, and assuming the likeness of God” (*The City of God* trans. Marcus Dods VIII.8, qtd. in Arnold 5). It is knowing and imitating God through “the aid of divine illumination” (Arnold 5). Milton’s definition of “sounder wisdom” in his *Proslution III* follows the same formula of the classical descriptions of right reason, combining the faculties of reasoning with moral actions (Arnold 18). Milton also follows his predecessors in identifying divinely inspired knowledge as greater than external discourse when he identifies, in *De Doctrina Christiana*, the internal guidance of the Holy Spirit and “the unwritten word” as “a more certain guide” than scripture alone (*Works* v.16 281, 279).³

So when Milton has Raphael differentiate discursive from intuitive reasoning, he is distinguishing

²For a more detailed debate on the extent to which Milton diverged from Ramus, see Duhamel and Fisher.

between a lower, human way of knowing that relies on external discourse and is more closely connected to logic, and the higher, more complete right reason that is intuitive, imaginative, and divinely inspired. This conception of reason influences the text of *Paradise Lost*. It is discussed by characters in the poem, it influences the depiction of the characters—Satan, for example, displays only discursive reasoning—and, crucially, it impacts how Milton presents his justification of the ways of God to men.

The most obvious way Milton advances his argument is with God's speech at the beginning of Book III, where the Father explicitly explains his own justice, goodness, and even mercy in regards to the Fall. God declares, "freely they stood who stood and fell who fell," and says that "if I foreknew / Foreknowledge had no influence on their fault / Which had no less proved certain unforeknown" (*PL* III.102, 117-119). Later on, he adds that "man shall not quite be lost but saved" and all those, not just an elect few, who are guided by God-given "umpire conscience" (that is, right reason) will be delivered (*PL* III.173-197). Milton thus uses God as an authoritative figure to express clearly and reasonably his Arminian beliefs of "conditional election, unlimited atonement, [and] the absolute freedom of the creaturely will" (Gregory 200-201). Omniscience and omnipresence, Milton's God is saying, are not equivalent to predetermination. These beliefs constitute the foundation of Milton's argument regarding why humanity should view God's ways, in particular as they relate to the Fall, as just.

However, Milton obviously did not believe this speech was enough to convince his readers of the justness of God's ways. If he had felt sanguine, as Donnelly has noted, the poem would "simply end at Book 3" (*Milton's* 102). Communicating the substance of an argument is not the same as communicating intuitive understanding and acceptance on a spiritual level. As Milton says in *Areopagitica*, a "man may be a heretic in the truth"; knowing something, and even believing in it, is meaningless without reason-based understanding (365). This is why God's speech does not, and cannot, stand alone as an argument. Furthermore, there is a tension in the discourse of Milton's God as he declares

that man has free will while nonetheless demonstrating his own foreknowledge. Below the surface there is an instability to the logic, an unconvincing portrait of an all-powerful being that declares in advance what others will supposedly freely do.

These passages in Book III are a form of divine self-revelation, but this revelation takes the form of an interrogative conversation (Donnelly, *Milton's* 101). According to Milton's differentiation between discursive and intuitive reason, this conversation is incomplete human reasoning, an external verbal discussion that, however important it may be, is of relatively lower degree because is not internal enlightenment. Of course, within the poem, this discussion is effectively an intuitive experience for the Son and the angels, because they receive direct enlightenment from God. However, regardless of how the Son and the angels perceive God's revelation in this passage, the argument the reader is presented with is the poet's portrayal of this communication, just as we are presented with a poetic representation of God, not God himself. Thus, it is this discursive reasoning that serves to justify God to the reader, and this interrogative conversation alone does not fit with Milton's preference for intuitive and imaginative reason. It is incomplete, unless it is viewed in the context of the whole poem, where Milton expands his argument beyond discursive speech to facilitate implicit reason in his readers so they might know and understand, and not just have been told of, God's justice.

In Book VI, Milton lifts human reason up into the realm of divine knowledge through temporal confusion and compression. Milton makes an imaginative argument about the nature of divine justice by elevating his readers closer to an omniscient perspective and a divine understanding of time. It is an argument of experience and discovery, providing some of the demonstrative proof Milton believed to be necessary to supplement his prophetic testimony. Furthermore, it is a form of right reason, of knowing and imitating God. It is literally, to return briefly to Augustine's definition of right reason, "assuming the likeness of God."

Elevating his readers to a divine perspective provides Milton with a unique and powerful tool to succeed in his argument because of the traditional theological meaning of the term "justification." In Christian religious thought, justification is the "process through which fallen mankind is either made or declared righteous in the sight of God" (Bryson 92). As

³ For a fuller discussion that interprets the entirety of *Paradise Lost*, and in particular the Fall of Adam and Eve, through the lens of Milton's own writings on right reason and logic, see Arnold.

Bryson points out, by claiming his purpose as justifying the ways of God to men, “Milton is appropriating and reversing the process through which Man is reconciled to God. Rather than reconciling Man to God, Man is reconciling God to Man” (92). Or rather, “the ways of God,” as Milton was not so prideful as to claim the ability to accuse and acquit God, but rather was trying to alter “the way in which God is conceived of by human beings” (Bryson 94).⁴ In Christian theology, the way humanity is reconciled to God is through Christ, through the person of God coming down to the human domain and living among us. So if Milton, as I am arguing, is elevating humanity’s perspective closer to a divine viewpoint, he is not only reversing whose ways are reconciled to whom, but also the major element by which this reconciliation takes place.

Theological “defenders of the doctrine of moral freedom,” like Milton, have long depended on the difference between the human experience of time and the divine eternity to reconcile free will with an omniscient, omnipotent God (Bedford 63). From the perspective of a human in time “there is causality, suspense, sequence” in the way we experience the world, but God exists independent of time, of causality or sequence (Bedford 74). In human time, foreknowledge of an event implies that the event is predestined to happen. However, from the point of view of God, foreknowledge is simple perception (as there is no before or after), and such knowledge does not in any way circumscribe the free choice of moral actors.

Paradise Lost stresses God’s place outside of time. The poet originally introduces us to his God by declaring that “past, present, future He beholds” “from His prospect high” (*PL* III.78,77). And if this simple discursive declaration is not enough to express the difference between the human and the divine experiences of time, Colie points out that contrasting God’s speech

in Book III, encompassing his view of the entirety of human history in just a few lines, with Adam’s view of history in an extensive sequential order that fills almost the entirety of Books XI and XII, allows the readers to experience such a disparity for themselves (132).

As postlapsarian humans, we are trapped in time, and the divine eternity is a cosmic mystery beyond the full comprehension of our mortal reason. Yet although I agree with Colie that God’s speech in Book III can help readers begin to understand something of the difference between a human viewpoint and an eternal one, I would argue that Milton’s understanding of intuitive logic empowers him to construct Book VI in a way that advances this goal much further. Whereas God’s words in Book III can be as problematic for readers as they are helpful, Book VI creates a unique sense of time that allows the poem to try to draw us nearer to experiencing that cosmic mystery, to go beyond our mortal scope and see what is inconceivable to the human eye. Since intuitive logic depends on personal experience leading to understanding, the poem creates a unique sense of time for the reader that mimics the divine perspective of eternity. After all, it “is in the medium of His eternity that God has foreknowledge” (Colie 128). Therefore, *Paradise Lost* brings us outside time to create a similar sense of foreknowledge. Modern scholarship has generally been very interested in the malleability of time throughout the entire poem, the way the present and the past are related, the blurring of chronology, and the sense of a unique “mythic time,” that is created in the poem (Welch 13). However, Book VI in particular manipulates its readers’ perception of time to help readers approach an omnipresent perspective.

Satan’s cannon, and Raphael’s aside to Adam about it, is one of the most striking examples of this in Book VI. As mentioned earlier, the reader foreknows the existence of the cannon before it is invented in the future by “Someone intent on mischief” (*PL* VI.503). Like Milton’s God, readers get to experience a moment outside of time where we know how an event transpires simultaneously before it occurs, while it occurs, and after it occurs. This temporal dislocation is compounded by the wording of Raphael’s aside. When he declares that “In future days...Someone intent on mischief or inspired / With dev’lish machination” will invent the cannon, the word “future” loses its normal function as a temporal marker (*PL* VI.502-504). The word simultaneously refers to Adam’s future, when mankind will create artillery, and to the seventeenth-century

⁴ Donnelly argues that because Milton “assert[s]” Providence, God’s goodness, before he declares that he will justify the ways of God to men, Bryson’s interpretation of “justification” as declaring God righteous before the eyes of man is not plausible (*Milton’s* 81-83). That is, if Providence is asserted, God is not apparently evil and thus does not need to be justified in this way. I contend, however, that there is, again, a distinction between asserting something and having it be understood. Post-lapsarian humanity doubts God, and even those who believe in divine Providence may struggle to reconcile God’s inherent goodness with what appears to fallen men as divine evil.

reader's present, when cannons are being commonly used in European warfare. It also refers to the past, as the "someone" who "with dev'lish machination" devised such an instrument was in fact, according to the poem, the devil himself, Satan. Thus, for this brief moment in the poem, sequential time collapses and the reader experiences the birth of the cannon from a perspective close to divine omnipresence.

Beyond this particularly striking instance, a sense of collapsed time is diffused throughout all of Book VI. Much of this temporal confusion comes from the fact that both the language and style of this section are reminiscent of the classical Greek and Roman epics. On the surface, this is unremarkable, as Milton was intentionally setting out to write a great Christian epic. However, unlike Milton, Raphael is not relating the War in Heaven to seventeenth-century Englishmen. He is telling it to Adam, a newly made man who has never seen battle and has never read any epic poetry. When Adam remarks innocently to Eve that, "So near grows death to life, whate'er death is," it is made clear that Adam does not intuitively understand all concepts, that he does not know the meanings of words he has not experienced (*PL* IV.425). Nevertheless, Raphael's narrative is full of military terms. He tosses into the story images like "spears and helmets...and shields," "chariot," and "battalion" without giving Adam any sort of context in which to place them (*PL* VI.83, VI.358, VI.534). He also uses epic conventions like the focus on duels between heroic individuals. Milton's readers would have appreciated the use of such conventions, and would have understood the martial words that described the battle, but Adam could not have.

Furthermore, Raphael is not simply describing the war exactly as it occurred. Instead, he explicitly says that to allow "human sense" to understand a conflict of unearthly spirits, he will have to equate "spiritual to corporal forms as may express them best" (*PL* V.565, V.573-574). In other words, he consciously chooses every word he speaks about the events in Heaven, and he is theoretically striving to make them meaningful to Adam. In truth, however, the story is designed for Milton's contemporaries, and would be confusing to Adam.

Yet in the text of the poem, this inevitable confusion on the part of Adam does not occur. Unlike earlier, when he was innocent of death, he seems to have no trouble understanding the violent images of conflict that he is presented with here. On the one hand,

this is due to the necessity of using martial terms in order to describe a battle. However, the poem exploits this necessity to condense and confuse time for the reader. This is made clear by the fact that Book VI seems designed not to allow readers to gloss over the fact that Adam should be unable to comprehend the language he is hearing. It is sprinkled with lines that deliberately draw attention to the fact Raphael is using figurative language to try to help Adam imagine the war. For example, right before he describes Satan's and Michael's fight, Raphael questions the ability of the human imagination to understand what an angel says:

[W]ho though with the tongue
Of angels can relate or to what things
Liken on Earth conspicuous that may lift
Human imagination to such height
Of godlike pow'r. . . .

(*PL* VI.297-301)

Raphael questions this human ability right before he uses words that Adam cannot comprehend. Similarly, Raphael finishes his narration of the war by repeating that he is "measuring things in Heav'n by things on Earth, / At thy [Adam's] request," drawing attention to the fact that he is ostensibly telling this story to warn Adam about Satan in a way that Adam can understand, but is in fact "measuring" by things that do not yet exist on Earth (*PL* VI.894-895). In this way the poem encourages its readers to think constantly about this tension between the ostensible audience for this story, a man who cannot understand it, and the hidden but real audience, the poem's readers. While reading a story about the past, they are encouraged to think about how that past is experienced simultaneously in the present (by Adam), and in the future (by those reading about Adam's experiences).

While Milton manipulates and blurs time for various purposes throughout the poem, not just in Book VI, the context of this particular book strongly implies that the narrator works to raise the reader, through this manipulation of time, to a divine point of view. Even though the action of Book V was already established to have been taking place in Heaven, Book VI nevertheless begins by making the readers feel as though we are entering Heaven alongside Abdiel, as he returns to God after his debate with Satan. The book opens as Abdiel "unpursued / Through Heav'n's wide champaign held his way till Morn...with rosy hand / Unbarred the gates

of light" (*PL* VI.1-4). On a literal level, Abdiel is already in Heaven, and the fact that he travels until he meets "the gates of light" is merely a poetic way of saying that he travelled until dawn. However, the poet's choice of words invokes the image of golden gates opening to invite the reader into Heaven. This sense of movement into a divine sphere is enhanced by the very next image we receive: the home of the rising light, which both Abdiel and the reader are approaching, is revealed to be "Within the Mount of God fast by His throne" (*PL* VI.5).

This opening image of light also helps prepare us for the way that the poem will proceed to try to collapse time in order to approach eternity. The image of the rising morning that leads us to God's throne recalls the poet's invocation to light, followed with his depiction of God on His throne, at the beginning of Book III. In the Christian tradition that Milton wrote in, "light, divinity, and time" have well-established symbolic relationships that he manipulates to create in the invocation to light "a poetic conflation of...time and eternity" (Cirillo 55). The parallels of the beginnings of Book III and VI, therefore, not only highlight the way in which the later book uses intuitive reason to elaborate on the discursive argument presented in Book III, they also anticipate the way that the poet will manipulate time in his depiction of the War in Heaven.

Just as this section of the poem begins by taking us into a divine space, it ends with the poet leaving Heaven. The very first words that begin Book VII are a request from the poet to his muse to "Descend from Heav'n" (*PL* VII.1). Following her "voice divine," he has been led "Into the Heav'n of Heav'ns," but now he wishes to return to earth (*PL* VII.2,13). The simplest meaning of these lines is as a reference to the fact that the previous book and half have been set in Heaven, as Raphael has described events taking place there. However, these lines recall the invocation at the beginning of Book III, when Milton describes a similar change of place (from Hell to Heaven rather than from Heaven to Earth), but in doing so identifies with Satan's perspective, with his journey up from Hell. They thus imply a similar attempt by the poet to experience and portray a superhuman perspective. In this case, they enhance the sense that the perspective in Book VI is closer to God's than in other sections of the poem.

This identification of the poet's voice with a divine point of view is further developed with his remark that he has "drawn empyreal air" (*PL* VII.14).

As a guest in Heaven, he breathed in empyreal air—and thus, implicitly, expelled it with his voice as he sung of the events in Book VI. This conclusion is confirmed by his humble line later in the invocation that, "More safe I sing with mortal voice unchanged"—implying that he was earlier singing less safely with a voice that was not mortal (*PL* VII.24). In Book VI, the poet speaks with a divine voice.

It is also interesting to note the placement of this invocation that brings the narrator (and thus the reader) away from a divine perspective. While it marks the end of Book VI and Raphael's description of the War in Heaven, it is not placed at the end of Raphael's narration of divine history. In Book VII, he will go on to tell Adam the story of God's creation of the world. This choice emphasizes that the War in Heaven in particular, rather than Raphael's history as a whole, is being told from a divine point of view.

This distinction between the poet's adoption of a divine voice and Raphael's narration is important to the reader's experience of divine omnipresence. Angels, although closer to God than humans, are in the Great Chain of Being, exist in time and thus do not share in God's eternity. It is significant, therefore, that Book VI is not entirely told from an angelic perspective, even though Raphael is narrating it to Adam. Although at times Raphael talks about the battle as one who was participating, mentioning at one point the moment when "our eyes" confronted Satan's cannon for the first time, elsewhere he describes events he could not have been privy to, such as Satan's speech before the gathering of rebel angels (*PL* VI.571).⁵ At other times the narrator, who is ostensibly Raphael, distinguishes himself from both groups, as when, at the end of the first day of fighting, he refers to the two groups as "Michael and his angels" (rather than using "we angels," or some other signifier that would identify himself with the group) and "Satan with his rebellious" (*PL* VI.411, 414). The narrative voice thus cannot simply represent Raphael's perspective, but rather is, at least part of the time, an observer of the action with a more omnipresent perspective than a single angel, limited by time, could have.

⁵This insight into the fallen angels' actions is not evidence that "the point of view which we share...is that of the fallen angels," as Miller tries to argue (8). The reader, with a more inclusive, more omniscient point of view, is able to view both perspectives while being contained by neither.

As a rebel angel, cut off from God, Satan in Book VI has no such access to divine eternity. He cannot comprehend what omniscience entails, as he proves when he argues to his rebel host that since they have survived the first day of combat, God, who they “till now / Omniscient thought” is found to be “fallible it seems / Of future” (*PL* VI.429-430, 428-429). Since God must have sent out troops that he “judged / Sufficient to subdue us,” Satan reasons, his knowledge of the future, and thus his power, must be limited (*PL* VI.426-427). Satan can see divinity only as an expression of physical power in the immediate present, and thus he misinterprets both God and the events he experiences.

In his limited viewpoint, he represents the postlapsarian human view of time. Speaking to his troops, he declares that the first day of battle has proved that God cannot defeat them because they “have sustained one day in doubtful fight / (And if one day, why not eternal days?)” (*PL* VI.423-424). By extrapolating the outcome of one day to “eternal days,” he interprets eternity to be simply a linear expansion of time to infinity, because he is trapped in time. He falls into the trap of seeing God’s omniscience and omnipresence as mere prescience of the future, that must either determine what will happen or be flawed. This is the human perspective of time that the poem is trying to lift us above. By depicting Satan in this way, Book VI helps us observe our human point of view from a perspective similar to the divine eternity, to experience intuitively how the human habit of trying to infer eternal justice and reason based solely on our temporal experience is as flawed and ridiculous as Satan’s attempt to infer endless days from a single day.

Of course, this connection between Satan and humanity raises the question: since we are temporal creatures, how can we be raised up to something approaching a divine perspective if eternity cannot be inferred from time? Are we actually able to catch a distorted glimpse of omnipresence and divine reason by exalting our thoughts with intuitive logic, or are we, like Satan, unable to do more than create a false image of eternity cobbled together from our human experience of time? Ultimately, of course, this is an impossible question to answer; however, one of Milton’s peculiar beliefs included in this poem offers a potential resolution of this problem. Milton insisted, unusually for his time, in both *Paradise Lost* and in *De Doctrina Christiana* that time passes, and has always

passed, even before creation, in heaven (Welch 5). This assertion is problematic, as it seems to challenge God’s eternity, his place outside of time. Whatever Milton’s reasons for this belief, scholars looking at its effect on the poem have tended to accommodate it as matter of narrative expediency and look at how it helps create the poem’s overall sense of mythic time (Bedford 71).

However, this conception of time does not necessarily limit Milton’s God’s timelessness. The first mention of this concept in *Paradise Lost* is in another one of Raphael’s little asides, when he remarks that “(For time, though in eternity, applied / To motion measures all things durable / By present, past and future)” (*PL* V.580-582). That is, Raphael is saying that even actions that occur in pre-creation eternity can be discussed as sequential events. That said, this parenthetical comment takes on a more complex meaning when viewed in the context of the rest of the poem. Later on, Raphael refers again to time and motion before he tells of how God created the universe, declaring, “Immediate are the acts of God, more swift / Than time or motion but to human ears / Cannot without process of speech be told / (So told as earthly notion can receive)” (*PL* VII.176-179). Together, these passages imply that while time does exist in pre-creation eternity, Milton’s God, and the eternity that is one of his inherent characteristics, is still outside of and unbound by that time. God is “more swift” than “time or motion”—time may pass in Milton’s Heaven and before creation, but God is only portrayed as bound by such limits in order to make his actions intelligible to Adam and the readers.

So Milton’s God is outside of time, even though time can be applied within his eternity to describe motion—that is, anything that happens, including God’s actions. This paradox may help explain how temporally locked humanity can approach an omnipresent perspective. Because time can be applied in eternity to measure the created world, we have the ability with our human speech and ears to collapse time and use that experience to try to measure the events in the world as they appear to divine eternity. We cannot entirely escape time, but by expanding the reach of time into the pre-created world, and by allowing it to apply to God’s eternity, Milton increases the degree to which humankind can understand God’s perspective intuitively.

As Book VI of the poem raises the reader beyond human time and human reason into the intuitive realm of divine time and reason, it destabilizes the

human perceptions of the paradoxes of free will and foreknowledge, as well as of omnibenevolence and mortal suffering. Through the narrative techniques the poem employs and its compression and confusion of time, the poem works to bring the readers to the point when we will be able to resolve intuitively the paradoxes and see with a God's-eye-view how these conflicting concepts can exist in harmony.

Milton's God is presented to the reader, from the very first moment of his appearance in Book III, as encompassing all space and time. Although the poem has God declare the coexistence of foreknowledge and humanity's free will, the logical problems that this paradox creates make it difficult to embrace this forceful assertion. Likewise, the reader's own experience of human suffering, as well as the depiction in the poem of the suffering that Adam and his post-lapsarian descendants will undergo, makes God's justice, as declared both by the poet and his image of God, equally hard to accept. No matter how Milton tries to express God as a narrative figure, "his entrance remains in many ways hollow" (Fallon Samuel 46). It seems that he cannot be drawn down into the level of human reason, at least not in a way that a reader can connect with (Fallon Samuel 46). Since drawing God into mortal temporality is not sufficient on its own, the poem turns to bringing its human readers up into a more omnipresent, more divine, perspective than they normally have access to through Book VI; *Paradise Lost* attempts to induce an intuitive understanding of God's justice.

The opening of Book VI, as it prepares us to enter Heaven and a divine sense of eternity, also anticipates the transition between a flawed human perspective and a more divine view that is closer to the truth. After the transitional description of a traveling angel, the first thing presented by the narrator is the very concrete, material image of "a cave / Within the Mount of God fast by His throne" (*PL* VII.4-5).⁶ The poem presents its readers with not merely physical objects, but objects that carry with them a great deal of material mass, piled on top of each other: a cave, a mountain, and a throne. However, it then immediately contradicts that sense of weight by connecting these physical substances with an image of light living within them. Even the darkness that lives inside them is light, as Raphael reveals when he mentions that that "darkness there might well / Seem twilight here" (*PL* VI.11-12). The cave, mountain, and the throne, although they appear at first

view to be hefty and material, are actually infused with the immateriality of heaven. Similarly, when "Morn" is described a few lines below the Mount of God, the poem first presents a weighty picture of the dawn "arrayed in gold" (*PL* VI.12). This material image of metal is flipped immediately with the first word of the next line: it is "arrayed in gold / Empyreal" (*PL* VI.13-14). We are once again presented with a flawed, material perspective of the world only to have to poem shift us into a proper, more divine viewpoint.

One of the aims of this movement into a divine perspective is to demonstrate how God's foreknowledge, though all-encompassing, is consistent with the free will of the angels and of Adam and Eve. While the poem compresses time to display the past, present, and future all at once, leaving no question as to how events will unfold, it is also simultaneously imbued with a sense of choice and uncertainty. With the angels, this is primarily present in the existence of Abdiel. Although Satan and his followers are "to swift destruction doomed," Abdiel, as the one faithful angel, represents the path they could have taken (*PL* V.907). His presence in the poem opens up the possibility that the other angels could also have chosen not to fall. Without him, the War in Heaven would be between Satan's "train" and the angels who served around the Mount of God, the opposing sides seemingly predetermined (*PL* V.767). But Abdiel confuses the battle lines, for he was one of the as-yet-unfallen Satan's train—he is present at Satan's first counsel and Satan calls him "seditious" (*PL* VI.152). He thus clearly fights on the side of the loyal angels by choice, not by chance or necessity. This sense of choice on the part of Abdiel is emphasized by the suggestion, at the beginning of Book VI, that he could have fallen with his comrades. When God's loyal angels see Abdiel return, they respond by applauding and celebrating him, joyful "that one / That of so many myriads fall'n—yet one! / —Returned not lost" (*PL* VI.23-25). The implication, especially because of the excited interlude "—yet one!—," is that his continued loyalty was not a predetermined conclusion. If Abdiel could choose freely not to fall, then Satan and his followers could have so chosen as well, an assumption that Raphael confirms at the end of the book when he

⁶ Although I argue for a very different interpretation of Book VI than Miller does, I am indebted to his "Images of Matter" for introducing me to a discussion of materiality in Book VI.

warns Adam that, “Firm they might have stood / Yet fell” (*PL* VI.911-912).

Even more important than Abdiel for the argument that God gave humans the freedom to choose to fall, is the persistent presence of “ifs” in the epic.⁷ In several places, Raphael discusses the potential fate of men. When discussing the differences between human and angelic reason, he mentions that “Time *may* come when men / With angels *may* participate” (*PL* V.493-4; emphasis added). He adds, “*perhaps* / Your bodies *may* turn all to spirit” and that men “*may* at choice / Here or in Heav’nly paradises dwell-- / *If* ye be found obedient” (V.496-501; emphasis added). Though Raphael has already received the knowledge from God in Book III that Adam and Eve will not “be found obedient,” he still presents a picture of the future full of potential, where humanity might not fall into sin.

It is not merely visions of a joyful future that Raphael presents to Adam in such an open-ended way. At the end of his story of the War in Heaven, Raphael commands Adam to be wary of Satan, who is plotting to cause him to fall, “Which would be all his solace and revenge” (*PL* VI.905). This uncertain presentation is unavoidable from a narrative standpoint (Raphael cannot simultaneously warn Adam away from an action and tell him that taking such an action is inevitable), but coming as it does at the very end of this narrative arc, it works with the other moments of uncertainty to suggest the possibility of alternate futures.

This intrusive sense of potential alternatives to what both God and the reader know to be true also shows up in the poet’s carefully ambiguous word choice. At the end of his invocation to his muse at the beginning of Book VII, he reminds the reader of what has just transpired and says that Raphael told Adam about the War in Heaven “lest the like befall / In Paradise to Adam or his race” (*PL* VII.44-45). That “or” is fascinating, because from a historical and a narrative standpoint, its inclusion makes little sense. From a historical standpoint, as the narrator knows, Adam will fall. Nowhere else in the poem is there the suggestion that even if Adam did not fall, his descendants might have

fallen instead. From a narrative point of view, there no need to use “or” because both writer and readers know Adam will fall. Furthermore, this “or” comes as the poet himself is explaining that Raphael’s motivation was to warn Adam to keep himself “or his race” from falling—which makes no sense, because Raphael already knew that Adam would fall. The purpose of the choice of this particular word—especially when “and” could have fit the meter just as well—therefore appears to be another instance of adding confusion and uncertainty to what the reader knows to be true. It adds choice and possibility even amidst clear foreknowledge.

However, the most important “if” in this respect comes at the moment when Satan invents the cannon. “Someone intent on mischief...*might* devise / Like instrument,” Raphael says, but only “*if* malice should abound” [emphasis added] (*PL* VI.503-505, 502). In this aside, then, time is compressed so that the cannon is invented and used before it exists, but it does not have to exist. The cannon will be built, the cannon has been built, the cannon is being built—but only *if* men fall. After all, since Raphael is not able to describe the actual events of heaven, but only “lik’ning spiritual to corporal forms,” the cannon would not exist in the War in Heaven if it had not been invented on earth (*PL* V.573). The foreknowledge that both God and the reader have about the cannon’s existence, although certain, does not require that it must exist just because it will. Foreknowledge and free choice coexist.

These moments that imply different paths that history might have taken cannot merely be deceptive, a way of fooling Adam about his fate, because they exist for the reader as much as for Adam—and some of them exist only in the narration that is given to the reader, not to Adam. They do not suggest (except, perhaps, to Adam) that men really will not fall, as this is a surety from the very first line of the poem, when the poet demands his muse sing “Of man’s first disobedience” (*PL* I.1). But they are moments that inspire *an intuitive experience* of how events of the world depend not on time, not on when God knows things, but on choice. Adam will fall, Adam has fallen, Adam is falling. Adam will choose, Adam chose, Adam is choosing. Events are not predetermined by foreknowledge, nor is free choice limited, because God’s foreknowledge is unbound from time—and, in Book VI, so is ours.

The way that Raphael describes God’s actions during the War in Heaven, and God’s language in Book VI, demonstrate how Milton’s God relates to action and

⁷ Herman has a fascinating chapter on the extensive use of the explicit and implied “or” in the poem (although he focuses on different moments of possibility than I do), and how it creates a strong sense of uncertainty. However, he focuses on how this uncertainty relates to Milton’s life and the politics of the poem, whereas I examine these uncertainties for their place in establishing creaturely freedom.

choice through the medium of His foreknowledge. God does interfere in the battle, even before he sends the Son to cast out the rebel angels. Raphael relates that God “From His stronghold of Heav’n high overruled / And limited their might”—that is, the might of the two warring factions so that they cannot destroy Heaven with their power (*PL* VI.228-229). On the one hand, this is an acknowledgement of both God’s knowledge and his power to control events. He knows that the rebel angels would destroy Heaven, and his power is so great as to make both sides weaker. On the other hand, although God is over-ruling and limiting the battle, he is still detached from it—he is not controlling the actions and individuals or affecting their choices, merely mitigating the effects of those choices. In the same way, he does not cause the fall of Adam and Eve, but by offering humankind grace, and sending his Son to become Christ, he limits and alleviates its effects.

Of course, this harmonization of freedom and foreknowledge in Book VI does not address the other major paradox Milton needed to settle in order to justify the ways of God to men. The anguish and pain of human existence are hard for human reason to reconcile with an omnibenevolent Christian God. Post-lapsarian humans live in a “world / Of woe and sorrow” (*PL* VIII.337-338). Milton laments the suffering he himself has experienced—his imprisonment and blindness—in the invocation that begins Book VII, having “fall’n on evil days...In darkness and with dangers compassed round” (*PL* VII.26-27). By bringing his readers closer to comprehending God’s experience of creation, appealing to intuitive reason to alleviate the flaws in the human ability to comprehend God, the poet attempts to help them understand how, despite the presence of evil and pain, God’s “mercy first and last shall brightest shine” (*PL* III.134).

Book VI reconciles these paradoxes by showing how relatively transient, minor, and inconsequential our suffering is from a divine point of view, while acknowledging how horrific the evils of the world are to us. In particular, the battles of Book VI carry relatively little weight, especially compared with the debate between Satan and Abdiel at the end of Book V. The war seems to have no stakes and no real purpose. Its most important consequence, the fall of the rebel angels, was determined before the war even began. Satan and his host have already turned away from God in Book V, and are “to swift destruction doomed” (*PL* V.907). Their martial rebellion only confirms their

willful defiance and separation from God; it does not cause it.

Nor are there any lasting physical consequences of the battle. None of the angels can die, and they heal all their wounds relatively quickly. War causes the rebels to experience pain for the first time, and they suffer just as humans suffer. After he fights with Abdiel, “Satan first knew pain / And writhed him to and fro convolved, so sore” (*PL* VI.327-328). However, his suffering does not last, and “th’ ethereal substance closed, / Not long divisible” because “spirits...Cannot but by annihilating die / Nor in their liquid texture mortal wound / Receive no more than can the fluid air” (*PL* VI.330-331, 344-349). Just as the reader is first presented with a deceptively material image before receiving the truer, more divine perspective of the Mount of God at the beginning of the poem, Satan’s injury is first presented from the painful perspective of human experience before it is revealed to be relatively inconsequential and harmless.

Likewise, although the warring angels do a great deal of damage to Heaven’s topography over the course of their fight, this temporary chaos of war is undone almost instantly at the command of the Son near the end of the poem. The war first brings “foul disorder” to the heavenly landscape as it is strewn with broken armor and overturned chariots, and later causes the very hills of heaven to be uprooted and “Hurl’d to and fro with jaculation dire” (*PL* VI.288, 665). But this damage, an exaggeration of the damage that results from human wars, proves to be as temporary and deceptive as the injuries of the angels. Before the book is over, the Son speaks a command and “th’ uprooted hills retired / Each to his place...Heav’n his wonted face renewed / And with fresh flow’rets hill and valley smiled.” (*PL* VI.781-784). What initially appears to be the horrific effects of war proves to be easily fixed and no cause for concern.

This absence of any sense of stakes in, or consequences of, the battle is enhanced by the image of angels flinging hills back and forth, which the narrative presents not as a fearsome display of power but as ridiculous. Raphael informs his listener that the angels “plucked the seated hills with all their load...Uplifting bore them in their hands” and that “hills amid the air encountered hills” (*PL* VI.644-646, 664). The choice of words like “plucked” and the description of flying hills filling the air, without any sense of harm, makes the angels less like mighty Titans than like Saturday-

morning cartoon characters dropping anvils on each other's heads.

This almost farcical impression comes to a climax with the way Satan and Belial toss puns back and forth as they use the cannons against the faithful angels. While elsewhere in the poem Milton uses puns to make serious points, the sheer density of them in these passages turns the solemn battle into a comedy routine.⁸ For example, Satan pretends to be proposing peaceful negotiation while actually commanding that the cannons be fired by asking his angels to “briefly touch what we propound – and loud,” making three puns with only seven words (*PL* VI.566-567) (Teskey VI.559-67n.). Even then, these passages could be read as a horrifying Satanic mockery of the suffering of his enemies, except that, once again, there are no long-term consequences, no lives lost or damage done by the cannons. They turn the War in Heaven into a joke.

If heavenly battle proves farcical, earthly war becomes a game. Raphael remarks during his narration of the battle, “War seemed a civil game / To this uproar” (*PL* VI.667). Explicitly, he says only that the heavenly war is so terrible that battles on earth pale in comparison, not that human war actually is like a game—except that in the moment it takes our eyes to pass over the enjambment between “game” and “To this,” he is saying exactly that. Like a game, the War in Heaven is comical and without real consequences. And earthly war, in turn, seems like “a civil game” compared even to that farcical conflict. While expressly acknowledging the horrors of war, this line also implies it should not be taken seriously in the grand scheme of things.

And this, perhaps, is why Milton's God does not seem to take it seriously. From a perspective that reads the war as consequential and intentionally serious, God's reaction to it seems rather cold and distant, even tyrannical. Although he has the strength to limit the might of the armies as he pleases, he makes no attempt to simply stop it. He knows both that the battle will solve nothing and that the Son can end the war whenever he chooses, but he allows it to drag on for three heavenly days. If war is as horrific and as full of suffering as it appears from a human perspective, these do not seem to be the decisions of a just and empathetic god.

⁸ Miller finds the puns such an “offensive” violation of “poetic decorum” that he declares that the rebel angels' speeches in these passages constitute “an abuse” of language (10).

However, from the perspective of an omnipresent, omniscient God, a perspective that *Paradise Lost* is inviting us to try and share, the ornate distance of these passages is appropriate to the actual effects of the war. From the point of view of eternity, the battle is a harmless game, not worth his interference beyond limiting its effects to make sure it stays harmless. Furthermore, although he did not cause the war, he knows that he can use it to “great purpose,” to honor his Son and “declare All power on Him transferred” (*PL* VI.671-678). He takes a misfortune, war, and twists it into something purposeful and fortunate. From the divine perspective of Milton's God, he interferes just enough to limit suffering and to ensure that a greater good will come of it.

Together, the lack of physical consequences in the battle, the almost-farcical impression of the war, and weirdly distant tone of God's reaction give a strangely hopeful representation of human suffering. Just as Satan misinterprets the fact that the rebellious angels were not instantly overpowered to mean that God must not be omniscient, humans misinterpret our immediate experience because we are not omniscient. From God's perspective, Book VI seems to be implying, human battles are unimportant, not because God is not benevolent, but because human suffering, no matter how horrific it seems at the time it occurs, is relatively transitory and minor. After all, the only real consequence of the War in Heaven is that the rebel angels are thrown into Hell, without chance of redemption. No matter how much misery post-lapsarian humans might endure, they can be, by God's grace, redeemed. From an omnipresent perspective like the one into which the reader is lifted in Book VI, the evils humans experience are as temporary as the wounds of the angels in the face of God's grace. In this way, the poem attempts to reconcile the paradox of evil and benevolence, and justify God's ways to suffering men.

Milton's ultimate success in this venture to induce a genuinely inspired insight that brings his readers closer to understanding God's justice lies in the fact that in order to produce this insight, he is trying to induce it artificially, bringing its status as a genuinely inspired insight into question. This is particularly significant because the insight in this situation, for him, should be divinely inspired, but instead it is Milton-inspired. He is trying to get human logic to understand divine reason, to lift up human understanding so that it can understand divine eternity, to make human

reason explain what only divine understanding can encompass. This attempt is made problematic not only by the question of how post-lapsarian humans can use right reason to approach God, as mentioned earlier, but by the paradox of trying to make human reason become more divine through the force of external human logic. Milton's fear of overstepping his boundaries, of questioning the source of his own inspiration, is already strongly present in the poem, and this substitution of himself and his poem for direct divine inspiration feeds into that concern even more. These issues are never resolved in the poem, but the very last lines of the poem might indicate a way that Milton was addressing them: Adam and Eve "hand in hand with wand'ring steps and slow / Through Eden took their solitary way" (*PL* XII.648-649). The interesting contradiction of "hand in hand" and "solitary" might point to Milton's recognition of the problem of how he was trying to guide his readers into individual and genuine insight. Milton hopes to lead his readers, hand in hand, as fellow post-lapsarian humans towards a closer understanding of what he believes to be God's reason and justice, but ultimately, each reader must come to his or her own individual understanding.

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Multiplexed Loop-mediated Isothermal Amplification of the 16S rRNA Gene for the Diagnosis of Neonatal Sepsis in Resource-limited Environments

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Abstract

Sepsis, or dysregulated inflammation caused by bacterial infection, places a disproportionately high burden on newborns in developing countries. This is due in part to a lack of diagnostic tools suitable for sustainable use in resource-limited nurseries. One potential vehicle for a new diagnostic assay is loop-mediated isothermal amplification (LAMP), a high-yield DNA amplification method. LAMP has previously been used to detect genes from single species of bacteria in blood serum samples to aid in sepsis diagnosis. LAMP could be adapted to detect a broad set of bacteria, while retaining a degree of specificity that allows clinicians to begin directed antimicrobial therapy. Described herein is the successful design of a novel group of oligonucleotide LAMP primer sets that specifically bind to regions of the 16S rRNA gene of four bacterial orders. These could provide clinicians with a two-step sepsis-diagnosis technique that would provide a result in only one hour.

Introduction

Sepsis, or systemic inflammatory response syndrome in the presence of bacterial infection, is a leading cause of newborn morbidity and mortality, with the burden especially evident in developing countries; sepsis kills more than 1.6 million newborns annually in these resource-limited environments (Turner et al., 2013). The standard therapy for treating neonatal sepsis is administration of an antibiotic regimen, following clinical diagnosis. However, diagnosis of neonatal sepsis is complicated by its non-specific clinical presentation (Piantino, Schreiber, Alexander, & Hageman, 2013). While diagnostic tools must be used to aid in confirming sepsis diagnosis, current tools are not suitable for sustainable use in resource-limited environments since many of them are too expensive, require too much infrastructure, or are labor-intensive and too complicated for practical clinical application (Seale, Mwaniki, Newton, & Berkley, 2009; Vergnano, Sharland, Kazembe, Mwansambo, & Heath, 2005). Without diagnostic tools, clinicians must resort to prolonged broad-spectrum antibiotic therapies, operating under the assumption that clinical abnormalities connote sepsis. The unwarranted use of these antimicrobials continues to contribute to the increase of antibiotic-resistant strains (Kuppala, Meinzen-Derr, Morrow, & Schibler, 2011; Piantino et al., 2013). In addition,

prolonged broad-spectrum antibiotic therapy can lead to adverse outcomes for the newborn, including increased risk of necrotizing enterocolitis (Cotton, 2009; Piantino et al., 2013). Diagnostic tools must be developed that are accessible to clinicians practicing in resource-limited environments, so that targeted antimicrobial therapy can be implemented. The ideal tool would be cost effective, simple to use, highly specific, and highly sensitive.

Background

Culture of bacteria from blood is the diagnostic gold standard for neonatal sepsis. Bacterial pathogens may be isolated and identified from a blood sample once incubated. However, blood culture requires a significant amount of infrastructure and trained lab personnel; thus, it is not practical for use in resource-limited settings (Simonsen, Anderson-Berry, Delair, & Davies, 2014). Additionally, blood cultures require taking at least 1mL of blood from the neonate. Removal of this significant amount of blood can lead to adverse effects (Piantino et al., 2013). Moreover, blood cultures take too long to give a definitive result; even the most advanced blood culture systems take at least 48 hours to yield a positive result (Raju, Bhat, Lewis, & Vandana, 2013). This waiting period necessitates administration of broad-spectrum antibiotics until the pathogen is identified, potentially leading to the previously mentioned adverse

outcomes. Therefore, a more rapid diagnostic test must be developed to quickly identify pathogens causing neonatal sepsis.

Recent novel approaches to aid in diagnosis of sepsis have decreased the amount of time required for a result. Simple laboratory tests such as C-reactive protein or white blood cell count can provide a rapid, sensitive result of biomarker levels (Piantino et al., 2013). These types of tests could potentially be used sustainably in resource-limited environments. However, these biomarkers are actually indicators of Systemic Inflammatory Response Syndrome (SIRS), which has a variety of causes and therefore a variety of treatments. These biomarkers often lead to false-positive sepsis diagnoses, since clinicians assume that SIRS connotes infection (Rajani & Philip, 2011). In fact, sepsis is defined as SIRS caused by an infection (Goldstein, Giroir, & Randolph, 2005). There are many other causes of SIRS that are not pathogenically based (Piantino et al., 2013). Thus, false-positive diagnosis of sepsis based on SIRS biomarkers can result in unnecessary antibiotic administration. A diagnostic test must be developed that is specific to bacterial infection so that appropriate treatment options can be considered.

One approach to specifically detect bacteria from blood serum samples is to target regions of the bacterial genome. Since the development of polymerase chain reaction (PCR), many diagnostic tests for bacterial infection have been developed that rapidly detect nucleic acid sequences specific to prokaryotes (Lu, Perng, Lee, & Wan, 2000). PCR involves using a heat-stable polymerase to rapidly replicate DNA, using two oligonucleotide primers to specify the replication region. One advantage of PCR is that the *Taq* polymerase employed can amplify large fragments (>1000bp) of DNA, allowing for the amplification of some whole genes (Wu et al., 2007). However, PCR must be used in combination with sequence-specific probes or further laboratory analysis to provide identity information when targeting a wide array of species. (Lu et al., 2000). Additionally, in order to denature the DNA for replication to occur, the sample must be repeatedly heated and cooled, a process called thermal cycling (Notomi et al., 2000). This process requires a complex thermocycler, which is inaccessible to many resource-limited clinics.

To overcome the burden of sophisticated laboratory equipment, Notomi and colleagues (2000) developed a new method for gene amplification called

loop-mediated isothermal amplification (LAMP). Since LAMP employs a *Bst* DNA polymerase that exhibits strand-displacement activity, there is no need to thermally cycle the sample. This negates the need for a complex thermocycler; the only equipment required to carry out LAMP reactions is a hot-water bath. Also, unlike PCR, LAMP utilizes four primers to recognize six distinct regions of target DNA sequence, resulting in greater amplification specificity. Several studies have shown the specificity of LAMP primers to detect individual bacterial species and closely related strains in less than one hour (Hill, Beriwal, Chandra, Paul, & Kapi, 2008; Huy et al., 2012; Kimura, Yanagisawa, Wachino, Shibayama, & Arakawa, 2013; Lim, Teh, & Thong, 2013; Seki et al., 2005). All of these studies targeted small regions of DNA that are unique to given species, since the *Bst* polymerase in LAMP can only amplify target sequences between 100-300 base pairs (Notomi et al., 2000). While a diagnostic test that detects a single species could be helpful in some circumstances, it would not be a comprehensive detection method for all sepsis-causing bacteria. The ideal diagnostic test in a resource-limited environment would provide a broad result of definitely sepsis-negative or definitely sepsis-positive, with some indication of the type of bacteria present.

One gene target that is highly characterized and conserved across prokaryotes is the 16S ribosomal RNA gene. The conserved regions along this gene are common targets in PCR, since primers can be designed that are common to almost all prokaryotes (Lu et al., 2000; Wu et al., 2007). Flanking the highly conserved regions along the 16S rRNA gene are nine regions that are hypervariable across species. These hypervariable regions have been the targets of several species-specific detection and identification methods (Chakravorty, Helb, Burday, Connell, & Alland, 2007). Since LAMP can amplify small regions of DNA, it could be possible to amplify regions of the 16S rRNA gene along the boundary of the hypervariable and conserved regions. This could allow for an amplification of DNA from a broader group of species, while retaining a certain level of specificity. Since no single hypervariable boundary region could distinguish all possible prokaryotes causing sepsis, multiple hypervariable/conserved boundaries could be targeted with separate primer sets to distinguish bacterial groups present.

To perform multiple LAMP reactions simultaneously, Fang and colleagues (2010; 2011) recently developed a

multiplexed microfluidic chip. Although these reactions targeted viral genes, the concept could be applied to isolate LAMP products from reactions targeted toward various hypervariable/conserved boundaries of the 16S rRNA gene. In order to detect amplification without complex equipment, visual detection with fluorescent reagents could be used. Tomita and colleagues (2008) have recently demonstrated that calcein can act as a fluorescent indicator of amplification, based on the generation of pyrophosphate ions. Herein is proposed a point-of-care multiplexed loop-mediated isothermal amplification system that targets the boundaries of hypervariable and conserved regions of the 16S rRNA gene in bacteria causing neonatal sepsis, utilizing a simple visual detection method based on fluorescence.

Materials and Methods

Selection of Target Sequences and Primer Design

To determine which bacterial species to target, a literature review was conducted to find the most common species identified in neonatal sepsis cases in resource-limited environments. The 16S rRNA genes of the most common strains of species contributing to neonatal sepsis cases were accessed from the NCBI GenBank (Benson, 2013). These genes were aligned using ClustalΩ multiple sequence alignment software (Goujon et al., 2010). Areas conserved across species were selected as target sequences. PrimerExplorer V4 software from Eiken Chemical Co. (Nogi, Japan) was used to generate novel oligonucleotide primer designs using the selected target sequences. Primer designs were run through a BLAST search against the prokaryote database to check for *in silico* specificity.

Culture of Bacteria

Eight species were used as representatives, two from each of the previously mentioned orders that include sepsis-causing bacteria. Plate cultures of *Bacillus cereus*, *Staphylococcus aureus*, *Escherichia coli*, *Klebsiella pneumoniae*, and *Pseudomonas aeruginosa* were obtained from the microbiology laboratory on the University of Arkansas campus. Lyophilized ampules of *Acinetobacter baumannii* (ATCC® 19606™), *Streptococcus agalactiae* (ATCC® 13813™), and *Enterococcus faecalis* (ATCC® 19433™) were obtained from the American Type Culture Collection

(Manassas, VA). Lyophilized *S. agalactiae* and *E. faecalis* specimens were resuspended in 6 mL of brain-heart infusion (BHI) broth (Difco™) and incubated at 37°C for 24 hours. Lyophilized *A. baumannii* specimen was resuspended in 6mL of LB broth (Difco™) and also incubated under the same conditions. Glycerol stocks of all species were prepared with 50% of the appropriate broth (*S. agalactiae* and *E. faecalis* in BHI, all others in LB) and 50% glycerol, and then stored at -80°C. Following preparation of glycerol stocks, all specimens were individually quadrant streaked onto either BHI or LB agar plates and incubated at 37°C for 24 hours to isolate individual colonies. Plates were then stored at 4°C until DNA isolation.

Genomic DNA Extraction

Bacterial cell walls were lysed using boiling lysis technique, as described by Iwamoto and colleagues (2003), in order to extract genomic DNA. Briefly, a single colony of each strain was collected using an inoculating loop and individually suspended in 100µL of boiling lysis buffer (2mM EDTA, 1.2% Triton X-100, and 20mM Tris•HCl [pH=8], stored at 4°C). Cell suspension lysis tubes were then placed in a boiling water bath at 100°C for 20 minutes. Lysates were stored at -20°C until use in LAMP optimization reactions. Crude lysates were used directly in LAMP reactions.

Optimization of LAMP Reaction Conditions

Initial LAMP reactions were carried out to determine optimal conditions using Loopamp DNA Amplification Kit and calcein-based Fluorescent Detection Reagent, both from Eiken Chemical Co. (Nogi, Japan). Products from LAMP reactions were viewed under UV light at 365nm then run on agarose gels to confirm primer binding and amplification. To find optimal visual fluorescence results, LAMP was repeated for three sets of positive and negative controls using varying Mn²⁺ concentrations.

Determining Limit of Detection

To find the minimum amount of DNA that the primers could specifically amplify, the *P. aeruginosa* DNA sample was used as a representative. *P. aeruginosa* crude DNA lysate was serially diluted in lysis buffer.

LAMP was performed using Pseudomonadales primers with the lower dilution samples of the series. Results were viewed visually under UV excitation and with agarose gel electrophoresis.

Finding Specificity, Sensitivity, PV(+), and PV(-) of Primers and Calcein

LAMP reactions were performed using the optimized LAMP conditions. Primer sets were tested with DNA samples from species within orders for which they were designed. In addition, primers were cross-reacted with DNA from a species within an opposing order. Sensitivity, specificity, positive predictive value [PV(+)], and negative predictive value [PV(-)] were determined according to Equations (1)-(4), based on Rajani and Philip (2011), for both simple visual fluorescence and agarose gel electrophoresis detection:

$$\text{Sensitivity} = \frac{\text{True Positives}}{(\text{True Positives} + \text{False Negatives})} \quad (1)$$

$$\text{Specificity} = \frac{\text{True Negatives}}{(\text{True Negatives} + \text{False Positives})} \quad (2)$$

$$\text{PV}(+) = \frac{\text{True Positives}}{(\text{True Positives} + \text{False Positives})} \quad (3)$$

$$\text{PV}(-) = \frac{\text{True Negatives}}{(\text{True Neagtives} + \text{False Positives})} \quad (4)$$

‘True positives’ for simple visual detection method constitute an *emission* of fluorescence when the primer set is tested with DNA for which it *is designed* (i.e. a positive result is obtained when expected); ‘true negatives’ are a *lack* of fluorescence when the primer set is tested with DNA for which it is *not designed*; ‘false positives’ are an *emission* of fluorescence when the primer set is tested with DNA for which it is *not designed*; and ‘false negatives’ are *lack* of fluorescence when the primer is tested with DNA for which it *is designed* (Rajani & Philip, 2011). For primer sensitivity, specificity, PV(+), and PV(-), the same definitions apply, with ladder-like streaks on agarose gel in lieu of fluorescence.

Results

Target Sequences Selected

Based on the study by Zaidi and colleagues (2009), the eight most-commonly identified isolates of sepsis-causing bacteria are listed in Table 1. Further investigation was conducted of review articles determining the strains of those species responsible for neonatal sepsis.

Order	Organism	% of Isolates from All Regions
Enterobacteriales	<i>Escherichia coli</i>	17.08
	<i>Klebsiella</i> species	13.49
	<i>Salmonella</i> species	4.78
Lactobacillales	Group B streptococci	8.06
	<i>Streptococcus pneumoniae</i>	5.67
Pseudomonadales	<i>Pseudomonas</i> species	7.17
	<i>Acinetobacter</i> species	4.20
Bacillales	<i>Staphylococcus aureus</i>	13.30

The 16S rRNA genes accessed from NCBI GenBank are listed in Table 2. Based on the phylogenies of species responsible for sepsis, it was determined that target sequences should be common to the order-level

Table 2. 16S rRNA Genes Accessed from NCBI GenBank, with strain or serovar of species listed with accession numbers.

Order	Species	Accession #
Bacillales	<i>Bacillus cereus</i> (ATCC 14893)	AJ310098
	<i>Listeria monocytogenes</i> str. 4b F2365	AE017262
	<i>Staphylococcus aureus</i> subs. aureus	CP000730
	<i>Staphylococcus epidermidis</i> RP62A	CP000029
Enterobacteriales	<i>Enterobacter sakazakii</i> ATCC (51329)	AY752937
	<i>Escherichia coli</i> O7:K1 CE10	CP003034
	<i>Klebsiella pneumoniae</i> K2	CP006648
	<i>Salmonella enterica</i> sero. enteritidis	DQ344532
	<i>Salmonella enterica</i> sero. typhimurium	DQ344533
Lactobacillales	<i>Enterococcus faecalis</i>	AF039902
	<i>Streptococcus agalactiae</i> sero. V	AE009948
	<i>Streptococcus pneumoniae</i> R6	AE007317
	<i>Streptococcus pyogenes</i> M1	AE004092
Pseudomonadales	<i>Acinetobacter baumannii</i>	X81667
	<i>Pseudomonas aeruginosa</i> #796	AB037553
	<i>Pseudomonas oryzae</i> str. WB2003S	AY850170

of classification. This resulted in four orders of bacteria to be separately targeted: Bacillales, Lactobacillales, Enterobacteriales, and Pseudomonadales.

To find regions conserved within an order, three to five dissimilar 16S rRNA gene sequences from the same order were aligned with ClustalΩ multiple sequence alignment software (Goujon et al., 2010). The consensus sequence from this first alignment was then aligned with two 16S rRNA gene sequences from each of the three opposing orders, since the phylogenies of the orders differed from one another

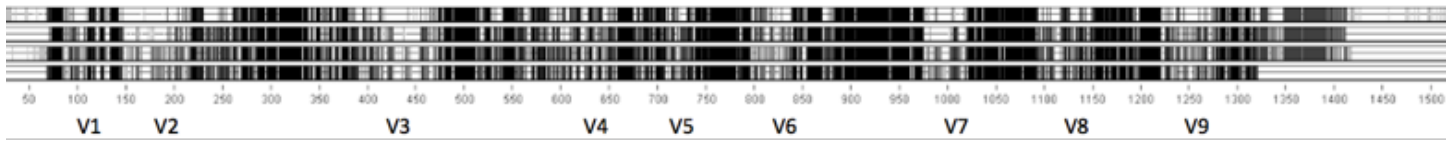


Figure 1. Conservation across consensus sequences of the 16S rRNA gene from the four bacterial orders of interest in neonatal sepsis. Darker = higher conservation. V1-V9 are the nine hypervariable regions.

Table 3. Final novel oligonucleotide primer designs, listed 5' → 3'.

Order	F3	B3	FIP	BIP
Bacillales	GGTGAGTAACACGTGGAT	TATGCATCGTTGCCTTGG	CATGCGGTTCAAATATTATCCGGT- AACCTACCTATAAGACTGGGAT	GAAAGACGGTCTTGCTGTAC- CGTTACCTTACCAACTAGCT
Lactobacillales	CTCGAAAGCGTGGGGAGC	GTAAGGTTCCTCGCGTTG	GGAAAGGGCCTAACACCTAGC- GGATTAGATACCTGGTAGTC	GGAGTACGACCGCAAGGTTG- TCGAATTAACACCATGCTC
Enterobacteriales	AAGACTGACGCTCAGG	CAGGTAAGGTTCCTCGCG	AGGGCACAACCTCAAATCG- GCAAACAGGATTAGATACCCT	GAGTACGGCCGCAAGGTTAA- TAAACCACATGCTCCACC
Pseudomonadales	AGGCCTACCAAGGCGAC	CTCCCAACTTAAAGTGCTTTA	TAGGAGTCTGGCCGTGTCT- CGTAACTGGTCTGAGAGG	GAGGCAGCAGTGGGGAATAT- CGAAGACCTTCTTCACACA

enough to be represented by only two species each. This alignment file was compared using Base-by-Base software (Hillary, Lin, & Upton, 2011) to view regions of the consensus sequence of the first alignment that are unique from the 16S rRNA gene of other orders. Unique regions of a conserved sequence were noted, and the process was repeated for the 16S rRNA genes of the remaining three orders. All four consensus sequences were aligned again and compared using Base-by-Base software to show the nine hypervariable regions along the 16S rRNA gene, seen in Figure 1.

Novel Oligonucleotide Primers Designed

Four novel oligonucleotide primer sets were designed using PrimerExplorer V4 software. Each set consisted of four primers: F3, B3, FIP (containing regions F1c and F2), and BIP (containing regions B1c and B2). Briefly, the consensus sequence of an order-specific alignment was used as a target DNA sequence. Mutations were introduced in the PrimerExplorer design window at positions on the target sequence where the consensus sequence was nonconserved across species within the order. This caused the primers to be assigned

accordingly, with the 3' end of internal primers F1c or B1c; 5' end of F2 or B2; and 5' end of F3 or B3 being the only primer regions overlaying the mutations (A Guide to LAMP Primer Designing). An example of this is shown in Figure 2. These mutation sites thus have minimal effect on primer specificity.

Using the Base-by-Base results as a reference, primers were then assigned locations unique to consensus sequences of a given order on the boundary of the hypervariable and conserved regions of the 16S rRNA gene. Therefore, primers bind to regions conserved within an order, but variable across orders. This process was repeated three times for each of 16S rRNA genes of the remaining orders. Each primer set design was then run in a BLAST search against prokaryotic genomes to confirm theoretical primer specificity for all species within the prescribed order. The final primer designs are listed in Table 3.

LAMP Confirmation and Cross Reactions: Primers Specifically Bind

For each LAMP optimization reaction: 40 pmol of FIP and BIP, 5 pmol of F3 and B3 from the prescribed primer set, 12.5 µL LAMP reaction mix (2x), 1 µL

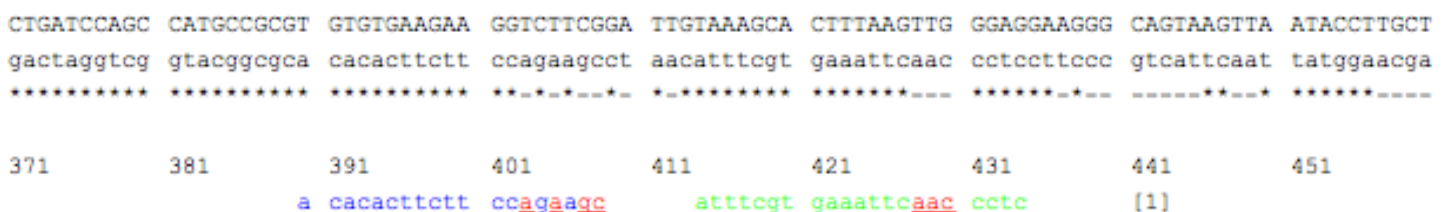


Figure 2. Sample of primer assignment to short sequence of conserved 16S rRNA gene across the order Pseudomonadales. The top sequence is conserved 16S rRNA gene. Dashes are introduced 'mutations', or areas of non-conservation within the Pseudomonadales order. Primer B3 (chartreuse) and region B2 of primer BIP (indigo) are assigned so mutations fall at the 5' ends, reducing the mutations' effect on binding. General B3 primer location was assigned to the edge of the hypervariable region (beginning at 401+).

Bst DNA polymerase, 2 μ L of the prescribed crude DNA lysate, 1 μ L Fluorescent Detection Reagent, and 4.5 μ L microbiology-grade H₂O were added to a sterile μ centrifuge tube, for a total reaction mixture of 25 μ L. For positive control, Loopamp positive control DNA and positive control primers were added to LAMP reagents in lieu of sample DNA and novel primers. Positive control primers from Eiken are specific to recombinant pDNA with HindIII insertion (provided in Loopamp Kit). For negative control, sample DNA template was replaced by water. Each reaction mixture was incubated in a 63°C water bath for 60 minutes. LAMP reactions were then terminated by incubation at 80°C for 2 minutes to inactivate the polymerase. This procedure was repeated, but with 6 μ L of Enterobacteriales primer set added to *E. coli* and *K. pneumoniae* reactions. In this second optimization reaction, the incubation temperature was increased to 65°C. Results of primer confirmation reactions, using optimized LAMP conditions, are shown in Figures

3-5. Amplification products were viewed under UV light then measured with a Qubit 2.0 fluorometer to find relative fluorescence units (RFU) in the green wavelength (500-525nm). Amount of fluorescence did not correspond to amount of DNA amplified, when compared with the products run on a 2.5% agarose gel. Positive fluorescence was not significantly higher than negative fluorescence. LAMP cross-reactions were also viewed under UV light and run on an agarose gel, see Figures 5 and 6.

Sensitivity, Specificity, PV(+), and PV(-)

The results of the LAMP confirmation and cross-reactions are summarized in the matrix in Figure 7. Calcein fluorescent probe statistically displayed theoretical 100% sensitivity, but very low specificity, between 0-67% depending on reaction. In addition, it displayed 100% PV(-), but varying PV(+) between 50-100%, depending on reaction. Each of the primer

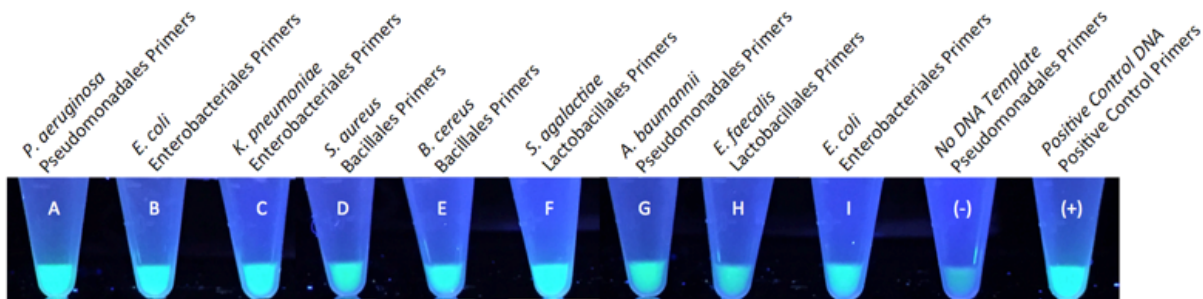


Figure 3. LAMP confirmation reactions: primer sets paired with DNA samples from species within orders for which they were designed. Visualized with UV lamp at 365nm.

Calcein Not a Reliable Fluorescent Probe Under these Conditions

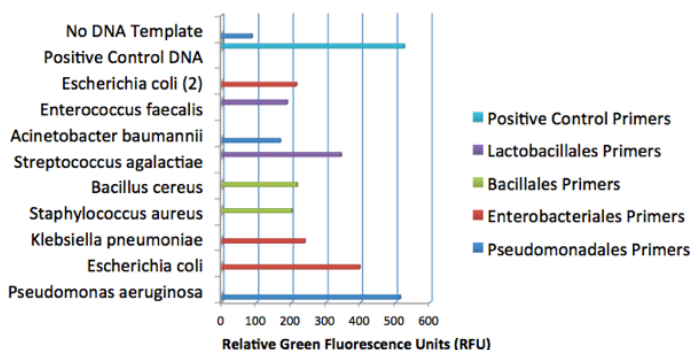


Figure 4. Relative fluorescence units of calcein probe following LAMP confirmation reactions. Positives are not significantly higher than negatives ($p=.05, n=10$). $\lambda_{excitation}=470nm$, $\lambda_{emission}=510nm$.

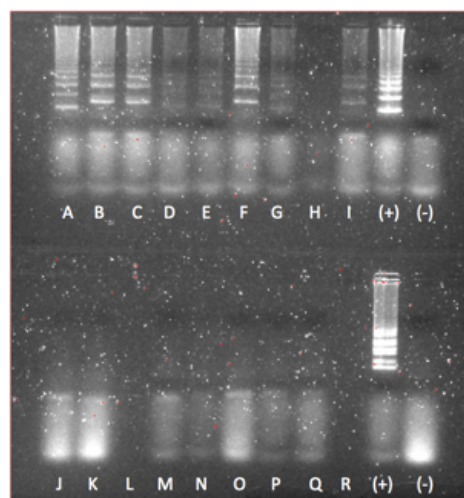


Figure 5. Electrophoresis of LAMP confirmation and cross-reactions. Lanes A-I: LAMP confirmation reaction products (no product added to lane H). Lanes J-Q: LAMP cross-reaction products (no product added to lanes L, R). Species labeling corresponds to that of Figures 3 and 6. Run on TBE/agarose (2.5%) gel at 100V for 40min.

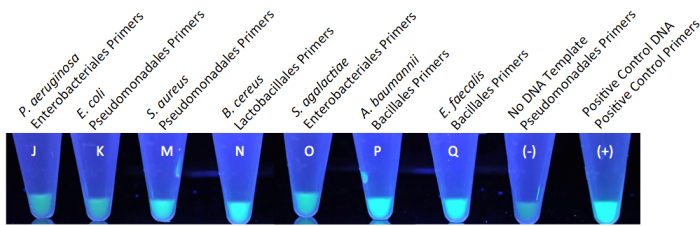


Figure 6. LAMP cross-reactions: primer sets paired with DNA from species of the closest-related order, or with a more phylogenetically distant order from the primer's designated order. Calcein non-specifically emits fluorescence, even with no amplification. Viewed under UV lamp at 365nm.

sets, however, displayed 100% sensitivity, specificity, PV(+), and PV(-) in this small trial—each primer set successfully bound to its target and no other targets.

Limit of Detection: LAMP More Efficient when Diluted

Crude DNA lysate from a single colony of *P. aeruginosa* was serially diluted to achieve a 1/10⁹ dilution. LAMP reaction products from the six most dilute *P. aeruginosa* DNA lysates and Pseudomonadales primers are shown in Figures 8-9. The primer set tested here displayed higher efficiency at lower theoretical concentrations, with more intense bands appearing on the agarose gel. Amount of fluorescence by calcein was not proportional to intensity of bands on agarose gel.

Discussion and Future Direction

Successful Novel Primer Design

Agarose gel electrophoresis results of LAMP

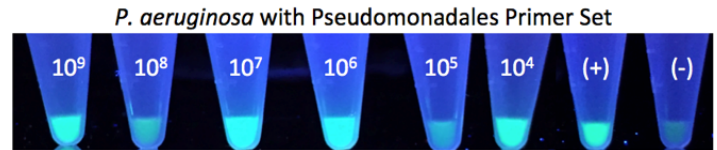


Figure 8. LAMP products of serial dilution reaction, showing dilution factor of crude DNA lysate from one colony. Viewed on UV lamp at 365nm.

products suggest that each primer set specifically binds to the target sequence for which it was assigned. These target sequences consist of hypervariable/conserved boundary regions of the 16S rRNA genes of species within four orders containing sepsis-causing bacteria. The electrophoresis results of both confirmation and cross-reactions showed non-specific streaks on the lower halves of lanes. The appearance of these streaks could be due to the presence of calcein in the loaded sample, which has similar excitation and emission wavelengths to SYBR[®] Safe, the DNA stain used in the electrophoresis. However, intensities of these lower streaks on the agarose gel were not consistent with intensity of fluorescence observed under UV. Alternatively, these streaks could be attributed to unreacted primers. The absence of these streaks in the more efficient LAMP reactions of the serial dilution products supports this hypothesis, where all primers would be utilized and incorporated into DNA product. Additionally, the presence of these streaks in negative cross-reactions and no-template controls supports that these bands are due to the presence of unreacted

Order	Primer Set	Pseudomonadales		Enterobacteriales		Bacillales		Lactobacillales		Positive Control	
		Fluorescence Result	Electrophoresis Result	Fluorescence Result	Electrophoresis Result	Fluorescence Result	Electrophoresis Result	Fluorescence Result	Electrophoresis Result	Fluorescence Result	Electrophoresis Result
Pseudomonadales	<i>Pseudomonas aeruginosa</i>	+	+	-	-						
Enterobacteriales	<i>Escherichia coli</i>	-	-	+	+						
Enterobacteriales	<i>Klebsiella pneumoniae</i>			+	+						
Bacillales	<i>Staphylococcus aureus</i>	+	-			+	+				
Bacillales	<i>Bacillus cereus</i>					+	+	+	-		
Lactobacillales	<i>Streptococcus agalactiae</i>			+	-			+	+		
Pseudomonadales	<i>Acinetobacter baumannii</i>	+	+			+	-				
Lactobacillales	<i>Enterococcus faecalis</i>					+	-	+	o		
Enterobacteriales	<i>Escherichia coli</i> (2)			+	+						
Positive Control	Positive Control DNA									+	+
Negative Control	No DNA Template	-	-								
	Sensitivity	1	1	1	1	1	1	1	1	1	1
	Specificity	0.66666667	1	0.5	1	0	1	0	1		
	PV(+)	0.66666667	1	0.75	1	0.5	1	0.66666667	1	1	1
	PV(-)	1	1	1	1	0	1	0	1		

Figure 7. LAMP results and statistical matrix for both calcein fluorescent probe and primer sets. Statistics referring to electrophoresis results are assumed to apply to primers themselves. Those referring to fluorescence results correspond to calcein characteristics. False positives are marked in red. The electrophoresis result from the *E. faecalis* reaction with Lactobacillales was excluded, since the LAMP product was not loaded properly into the gel.

primers. Positive results in agarose gels appear as dense ladder-like streaks from the top well down, due to the formation of varied-molecular weight stem-loop DNA produced from the *Bst* DNA polymerase (Notomi et al., 2000). These products are not visible in cross-reactions, since primers do not bind to target sequences, and *Bst* polymerase does not synthesize any new DNA. Very high levels of specificity, sensitivity, PV(+), and PV (-) for these primer sets are promising. However, these were calculated from a small data set, and should be re-evaluated using a larger breadth of species for both confirmation and cross-reactions.

Agarose gel electrophoresis results of LAMP products from serial dilutions of *P. aeruginosa* DNA lysate showed successful primer binding, even with template DNA at a $1/10^9$ dilution. It is interesting to note that the electrophoresis products of these serial-dilution amplifications suggest that the reaction was more efficient with less initial target DNA. The presence of the most intense ladder-like streak on the 10^9 lane

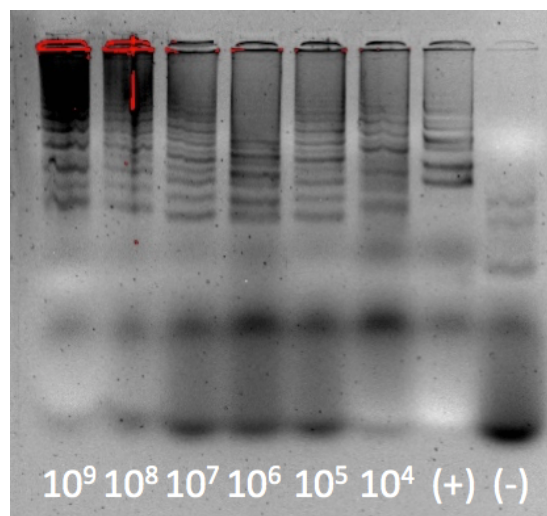


Figure 9. Electrophoresis of LAMP products for limit of detection of Pseudomonadales primer set, labeled with dilution factor of DNA lysate from one colony of *Pseudomonas aeruginosa*. Positive control: pDNA with HindIII insertion. Negative control: no DNA template. Run on TBE/agarose gel at 100V for 40 min.

of Figure 9 suggests that DNA amplification was most efficient with the most dilute DNA lysate. This is surprising, given the method of serial dilution. Dilution of the lysate following the boiling lysis prevents lysis bias, where lower concentrations of cells would be more efficiently lysed. The increased efficiency could be attributed to less non-specific binding of individual primers to other regions on the *P. aeruginosa* genome, freeing up primers to bind to the target sequence. Limits of detection for the other primer sets should be determined in the future.

Potential of Primers for Clinical Application

While many other LAMP primers have been designed to detect individual sepsis-causing species, no broad-spectrum LAMP primers have been developed. Large groups of bacteria are amplified by the primers described herein, which could provide a broad diagnostic method for sepsis-positive or sepsis-negative neonates. Though PCR primers can also amplify the 16S rRNA gene for bacteria, they provide no insight regarding the identity of the species present (Lu, 2000). Further laboratory analysis or additional probes are required to gain any indication of the type of bacteria present, since the PCR universal primers amplify the entire 16S rRNA gene for most bacteria (Chakravorty et al., 2007). In contrast, the novel LAMP primer sets described here are specific to four orders of bacteria. Future work should include clinical testing of samples compared with the gold standard of blood culture to confirm primer specificity. If these primer sets are separated and simultaneous reactions are carried out, clinicians could know the identity of any bacteria present in serum samples down to the order-level—enough information to begin a targeted antimicrobial regimen (Simonsen et al., 2014). This information could be attained in under an hour, with as little infrastructure as a hot-water bath and simple UV light, provided the specificity of the fluorescent reagent. However, simple visual detection with calcein fluorescent reagent did not correspond to the results of agarose gel electrophoresis.

Non-specific Fluorescence of Calcein Probe

Calcein should only exhibit fluorescent activity when an amplification reaction occurs; however, fluorescence was detected in many products not containing any amplified DNA, as shown by agarose gel electrophoresis. This could be due to the presence of metal chelators in the reaction solution, which would remove the Mn^{2+} quencher from calcein. These chelators, such as EDTA, could be present from the crude bacterial lysates used as DNA targets. Fluorescence was not consistent over similar reaction conditions, however, and non-specific results were detected each time. In addition, negative controls still emitted some fluorescence, where the intensity was not significantly different from positive reactions ($p=.05$, $n=10$). Therefore, calcein is an unreliable fluorescent indicator of amplification reactions in this circumstance.

Other avenues of visual fluorescent detection of DNA should be investigated. LAMP reactions should be repeated using SYTO-82 DNA stain. Initially it was hypothesized that SYTO-82 may result in non-specific fluorescence due to the presence of genomic DNA. However, given the magnitude of amplification product from LAMP reactions, it is estimated that there would be a significant difference in fluorescence emission intensities from amplified DNA (positive results) from non-amplified genomic DNA (negative results) stained with SYTO-82. If successful, SYTO-82 could be used as an indicator of simultaneous amplification reactions on a multiplexed microfluidic chip.

Potential for Multiplexing Reaction

To differentiate the orders present in a clinical serum sample, the four novel primer sets could be separated in distinct channels in a microfluidic chip. The LAMP reagents and lysed serum sample could be loaded into a central filling chamber that equally distributes the solution across the separate channels. A μ LAMP chip designed by a student colleague could facilitate these multiplexed reactions, pending the successful casting of PDMS replicas from 3-D printed molds. If successful, clinicians could use the μ LAMP chip as a two-step detection method—one step to expose the bacterial DNA and one step to load LAMP reagents—for diagnosing neonatal sepsis in about an hour. The successful design of novel oligonucleotide primers warrants further investigation into the visual detection mechanisms of the reaction.

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Terahertz Imaging Platform to Characterize the Growth of *In-Vitro* Breast Tumors

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Abstract

This study aimed at evaluating the ideal plating method and density for imaging with the terahertz (THz) spectrometer. In this study, different methods were used to grow in-vitro tumors using the 4T1 cell line. Here, attempts to grow breast tumors in-vitro were conducted. Results were produced in two environments, flat-bottomed plates and round-bottomed multiwell plates. The second method allowed for faster clumping and increased cell aggregation, producing tumors up to 7mm. Terahertz spectroscopy produced images that correlated well to photomicrographs taken of the in-vitro tumors. This methodology shows great promise for providing a reliable, parameter-controlled source of in-vitro breast tumors for research needs on breast tumor margins.

Introduction

Because breast cancer is the most common malignant disease in women (Weigelt et al., 2005), research to develop more effective treatments is extremely important. For the year 2015, the American Cancer Society predicts 234,190 new cases of breast cancer will be diagnosed and 40,730 deaths will occur (American Cancer Society, 2015). This statistic demonstrates an increase, rather than a decrease, in new cases of breast cancer from the year 2011. Routine screenings for breast cancer have become common practice in the United States since the 1960s, and x-ray mammography has been the primary method used (Hassan & El-Shenawee, 2011). The use of x-ray mammography comes with disadvantages; as a result, alternatives are being sought. For example, x-rays pose a health risk due to ionizing capabilities which are known to produce free radicals, break and form chemical bonds, and damage vital molecules such as deoxyribonucleic acid, ribonucleic acid, and proteins (Office of Environmental Health and Safety, 2015). This type of cell damage can lead to an increase in cancer development (Alberts et al., 2002), thereby compounding the problem. Additionally, the inaccuracy of x-ray mammography is high, ranging from 4% to 34% (Huynh et al., 1998). Magnetic resonance imaging (MRI) exists as an alternative to x-rays, but only for high-risk patients due to its expensive operating costs and its limited ability to distinguish between normal cells and cancerous cells, oftentimes leading to over-diagnosis (Hassan & El-Shenawee, 2011).

Removing the cancer before metastasis is also crucial, because metastases in the lymph nodes and other vital organs such as the lungs, liver, brain, and bones cause most of the complications linked to breast cancer, including death (Fantozzi & Christofori, 2006). During a lumpectomy to remove small tumors, a region surrounding the tumor, called the margin (Fig. 1) (Breastcancer.org, 2015) and (Bowman et al., 2015), is removed. A pathologist then examines the margin for cancerous cells. To maintain the best cosmetic appearance, it is important to differentiate between cancer, healthy fibrous and glandular tissue immediately surrounding the tumor, and healthy fatty breast tissue (Bowman et al., 2015). A positive margin, requiring a second surgery, demonstrates that cancer has extended across the edge of the excised tumor. The two other classifications do not require secondary surgery. The first of these is a close margin denoting cancer within 2mm of the tumor's edge and the second is a negative margin, where no cancer is detected within 2mm of the border. Positive margins not only cause cosmetic and emotional damage for the patient, but they are also very expensive due to the time and resources needed to carry out a second surgery. Research studies indicate that positive margins are detected 20-40% of the time (Pleijhuis et al., 2009).

A new medical imaging modality, available at the University of Arkansas, uses THz waves to develop high-resolution images and characterization of human breast tumors fixed in formalin and embedded in paraffin. THz waves measure from one mm to one μm , between infrared light and microwaves. Their

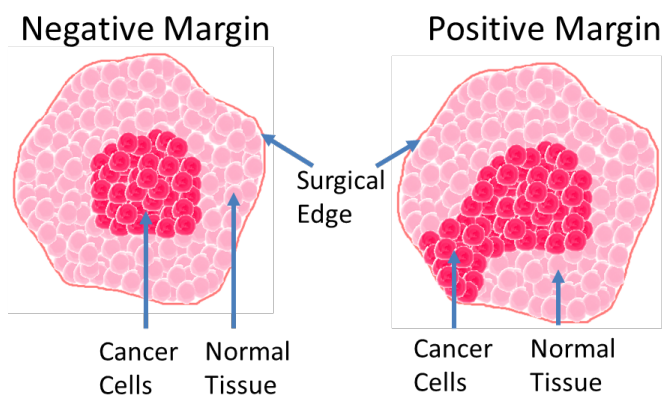


Figure 1. Animations of positive and negative margins (Breast Cancer Organization).

wavelengths have the medical advantage of possessing energy levels lower than the levels in x-rays that ionize cells (Burford et al., 2014). THz wavelengths have another advantage in that they are shorter than millimeter waves and therefore, generate higher image resolution while still penetrating the sample (Jepsen et al., 1996). Finally, THz waves produce limited levels of scattering in biological materials due to their longer wavelengths, instead of the strong scattering characteristic of near-infrared and visible wavelengths. Photons are scattered more strongly by biological materials because their size matches the incident wavelength (Wilmink & Grundt, 2011). For analysis, a THz pulse is emitted and strikes the sample, then a receiving antenna measures the intensity of the reflection off of the sample (Burford et al., 2014).

To further examine this new imaging system in a laboratory environment, attempts to grow breast tumors in-vitro were conducted. Current methods drip cells from an upper surface (Sato et al., 1977) or use multilevel chambers to pass the cells from an upper chamber, through a small pore, and into a bottom chamber where they are suspended from the roof (Dimri et al., 2007). The materials used in these methods are expensive and make the growth of multiple tumors unrealistic for most laboratories. Replacing these materials with agar gel and other commonly used substances could provide a reliable, parameter-controlled source of in-vitro tumors.

The cell line chosen for this experiment was the 4T1 mammary carcinoma cell line produced in BALB/cfC3H mice and first grown at the Karmanos Cancer Institute by Fred Miller (Tao et al., 2008). This cell line was chosen due to its tumorigenicity and invasivity. Furthermore, the 4T1 cell line is an ideal candidate

for studying human breast cancer because metastasis occurs randomly from the initial tumor, the cells are transplantable so they can also be grown in *in vivo* environments, and its spread to the lymph nodes and other organs is similar to the metastasis in human breast cancer (Pulaski & Ostrand-Rosenberg, 2001).

Methods and Materials

10% FBS DMEM media and 4T1 cell culture

A 10% FBS DMEM media was made from 50mL Fetal Bovine Serum, 5mL Penicillin-Streptomycin solution, 5mL L-glutamine, and 440mL Hyclone Dulbecco's High Glucose Modified Eagles Medium (DMEM). The 4T1 cell line was obtained from Dr. David Zaharoff's lab at the University of Arkansas. They were cultured in the 10% FBS DMEM media and incubated in a humidified environment at 37° C and washed with Phosphate Buffered Saline (PBS) every two-three days. Each week, the cells were passaged and two-three million cells were redistributed to a new flask with fresh media.

Flat-bottomed plates

Each 60mm flat-bottomed culture plate was lined with 4mL of agar gel made from 2mL of 10% FBS DMEM media, 1.33mL of 1.8% Noble Agar, and .67mL of sterile water. After setting for ten minutes, a mix of 2.67mL of the 4T1 cell solution and 1.33mL of 1.8% Noble Agar was added on top of the gel. Once the gel set, 2mL of media was added on top and the plates were incubated for two nights. The plates were viewed under an optical microscope every two or three days for four weeks. Fresh media was added to the plates to provide a new nutrient source for the cells.

Round-bottom multiwell plates

One percent agar was prepared from 40mL sterile water and .4g Difco Noble Agar, then autoclaved for fifteen minutes at 115°C to sterilize the solution. Fifty µL of agar was pipetted into each well of the first four rows (A-D) of a 96 well ultra-low attachment multiwell plate. The plate was constantly rotated as the gel cooled to ensure an even layer of gel coated the well while retaining the curved bottom surface. It was then overlaid with 10µL of 10% FBS DMEM media to

prevent the gel from drying out and allowed to incubate overnight. 150,000 cells were added to Row A, 300,000 were added to Row B, 600,000 were added to Row C, and 1.2 million were added to Row D. The 10% FBS DMEM media that contained the cells was changed every two to three days to continue providing nutrients.

Preparation of the samples

Multiple methods were used to prepare the samples for the THz spectroscopy and imaging system. Some of the flat-bottomed plates were fixed using a 2% paraformaldehyde solution while others were left as controls. Tissue Tek® O.C.T. Compound (OCT) was added to both samples in a plastic form to ensure optimal cutting temperature once frozen. Both types of plates were frozen and kept at -24°C. Using a Leica CM1860 cryostat, the samples were sectioned at a thickness of 20 µm.

The round-bottomed multiwell plate was frozen at -24° Celsius. Three samples with a starting cell count of 600,000 cells were sectioned with thicknesses of 40µm, 45µm, and 100µm and exposed to the air overnight to allow water molecules to evaporate from the OCT and gel before imaging. Another sample with a starting count of 1.2 million cells was sliced to a thickness of 40µm and placed directly on the viewing plate, preventing evaporation. A sample with a starting cell count of 150,000 cells was thawed, transferred directly to the viewing plate, and dried.

All samples were imaged using the TPS Spectra 3000 terahertz spectrometer from Teraview and analyzed using the TVL Imaging Suite (software package of the system).

Results

Flat-bottomed plates

The spheroids grown in the flat-bottomed plates grew to a maximum size of 200 µm with a maximum center of 75 µm (Fig. 2). They were completely encased in the agar gel base and were easily sectioned due to the gel.

In order to obtain THz images, the spheroids were embedded in OCT compound. Imaging these samples using the THz system did not show any field significant reflection from the spheroid grown in flat-bottomed plates. As shown in Figure 3, the THz image

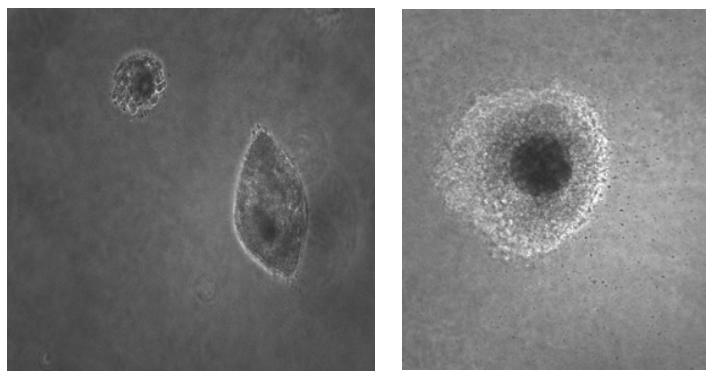


Figure 2. Photomicrographs of spheroids from flat-bottomed plates. Spheroids were immersed within the gel and imaged at 4× magnification.

demonstrates almost uniform reflection off the entire surface of the sample. The reflection imaging module of the THz system is shown in Figure 4.

Round-bottom multiwell plates

Wells containing over 150,000 cells appeared crowded with no areas of increased cell concentration or spheroid formation. The samples obtained from the 600,000 wells were difficult to section and the gel appeared very brittle and fragmented. Upon slicing, the gel containing the sample would flake and disintegrate as it rolled off the sample block or came into contact with the metal on the cryostat machine. Thicker sections were taken to obtain a solid sample without flaking. THz viewing showed no positive reflection where the tumor should have been. The 40µm slice showed the same reflection in the center of the OCT, where the tumor should have been, as the

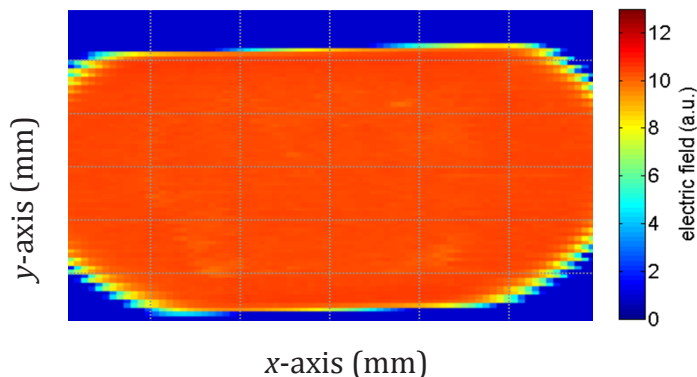


Figure 3. Cross section of 4T1 spheroids from flat-bottomed plate. The sample was embedded in agar gel and OCT compound. A 20µm thick section was cut and imaged using the THz spectroscopy and imaging system.

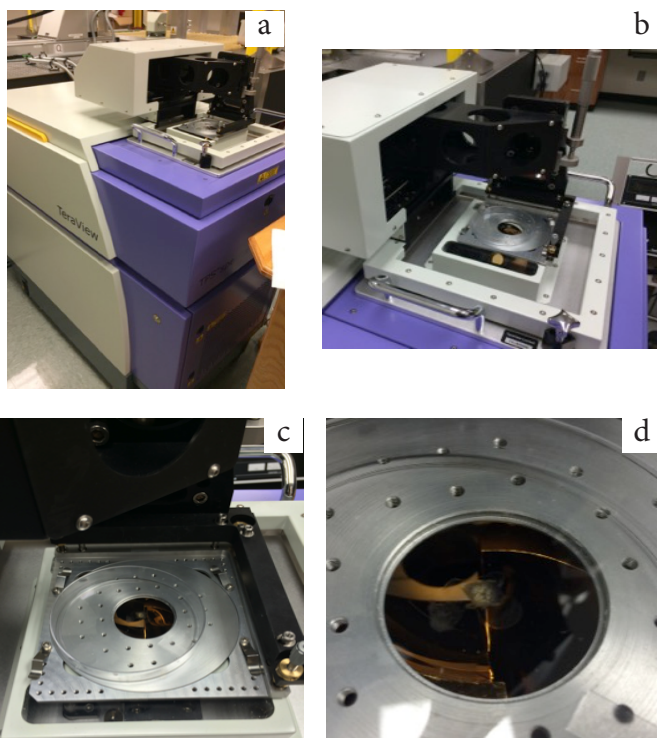


Figure 4. Images of the TPS Spectra 3000 spectrometer a) shows the core system as a whole, b) shows more detail of the reflection imaging module, c) shows the placement of samples within the viewing window, and d) shows a close-up of a potential sample.

area that had only the viewing plate (Fig. 5). The same was the case for the 45 μ m (Fig. 6) and 100 μ m (Fig. 7) slices. The bright blue spot on the 100 μ m slice was observed as a bubble (Fig. 7).

The sample from the well, beginning with 1.2 million cells, again disintegrated during sectioning and only showed a single reflection off the center of the viewing plate. No spheroids or concentrations of cells were present (Fig. 8).

Wells with 150,000 starting cells produced spheroids centered in the middle with multiple tighter spheroids around the edges (Fig. 9). Transportation of the samples to the viewing plates showed the tumors were embedded into the gel, but it is not clear how deep.

The colors seen on the THz images are assigned arbitrarily to best show the difference in reflections that are given off because the original image is black and white. THz spectroscopy showed a difference in reflection between the media that contained the sample and the tumors as a whole. In Figure 10a, the medium blue area surrounding the brighter blue shows a negative reflection, but one with a lower magnitude than the viewing plate. The brightest blue shows a positive reflection while the darkest blue shows a negative

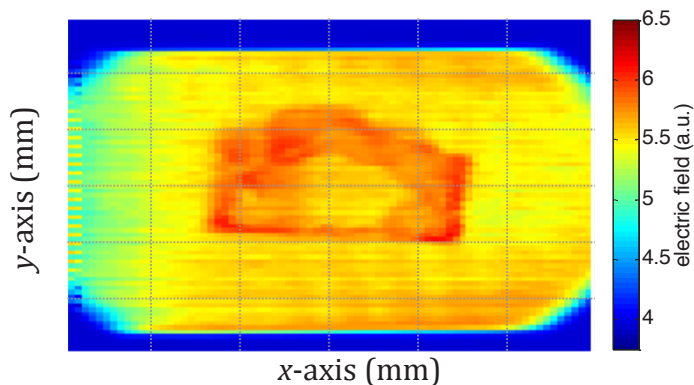


Figure 5. Cross section of 4T1 spheroid from 600,000 starting cells. The sample was embedded in agar gel and surrounded by OCT Compound. A 40 μ m thick section was cut and imaged using the THz spectroscopy and imaging system. The yellow area, where the tumor should have been, within the red area indicates a lack of cancer cells.

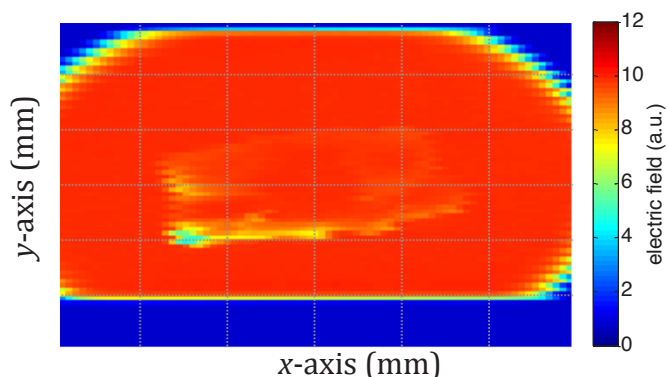


Figure 6. Cross section of 4T1 spheroid from 600,000 starting cells. The sample was embedded in agar gel and surrounded by OCT Compound. A 45 μ m thick section was cut and imaged using the THz spectroscopy and imaging system. The uniform reflectance across the sample indicates a lack of cancer cells.

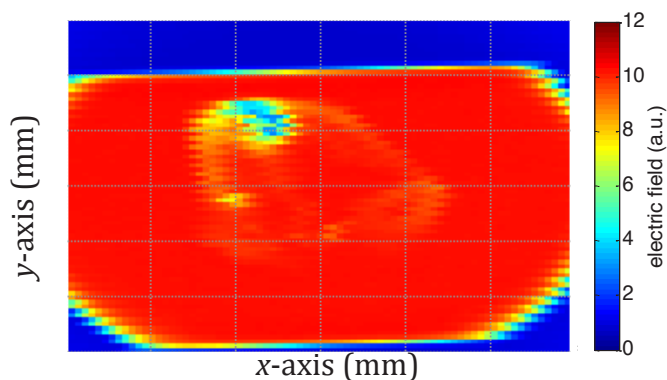


Figure 7. Cross section of 4T1 spheroid from 600,000 starting cells. The sample was embedded in agar gel and surrounded by OCT Compound. A 100 μ m thick section was cut and imaged using the THz spectroscopy and imaging system. The uniform reflectance across the sample indicates a lack of cancer cells, as the blue portion was a bubble.

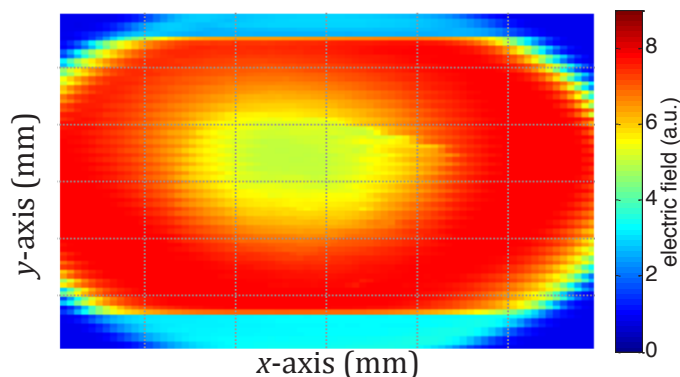


Figure 8. Cross section of 4T1 spheroid from 1,200,000 starting cells. The sample was embedded in agar gel and surrounded by OCT Compound. A 40 thick section was cut and imaged using the THz spectroscopy imaging system. The single reflectance in the center of the image corresponds to only OCT compound and indicates a lack of a tumor.

reflection. The area of positive reflection corresponded to the area of high cell density on the microphotograph and the negative reflection responded to the area of low density. The tumor measured about 5mm in diameter.

Figure 11a shows similar results with the central

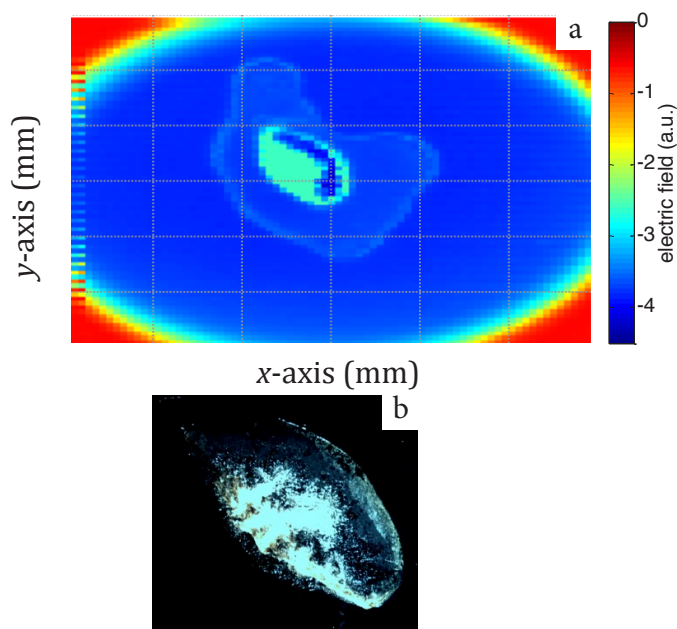


Figure 10. THz cross sections of 4T1 spheroids from 150,000 starting cells, first sample. The sample was embedded in agar gel and processed in its entirety using the THz spectrometer and imaged at 4x magnification. Figure a, shows positive reflection in the bright blue area and negative reflection in the darkest blue area, corresponding to the area of highest density and area of low density in the microphotograph shown in Figure b. The image was taken of the entire sample in a petri dish.

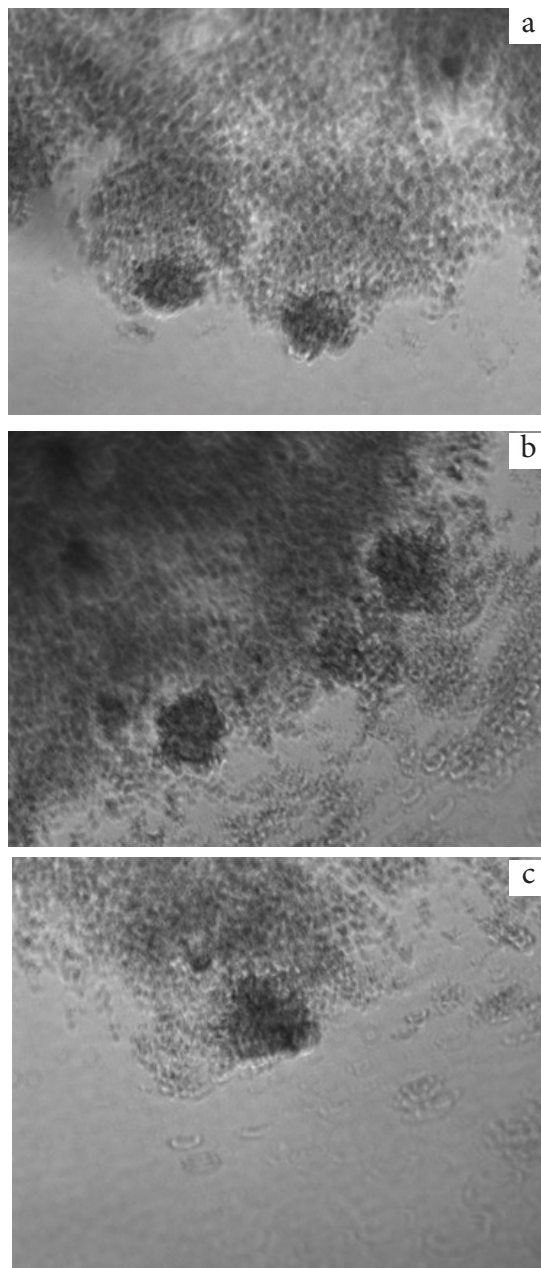


Figure 9. Photomicrographs of spheroids at 10x magnification from round-bottomed multiwell plate. The cells and spheroids were embedded into the gel base layer and the initial cell count was 150,000. Figures a, b, and c represent the spheroids clusters taken at different position of the sample.

red area exhibiting a positive reflection, the darkest blue area showing a negative reflection, and the yellow area around it demonstrating a negative reflection with a lower magnitude than the viewing plate. The red area correlated to the area of high density on the microphotograph and the dark blue correlated to the area of low density. Approximate size of the tumor was 7mm in diameter.

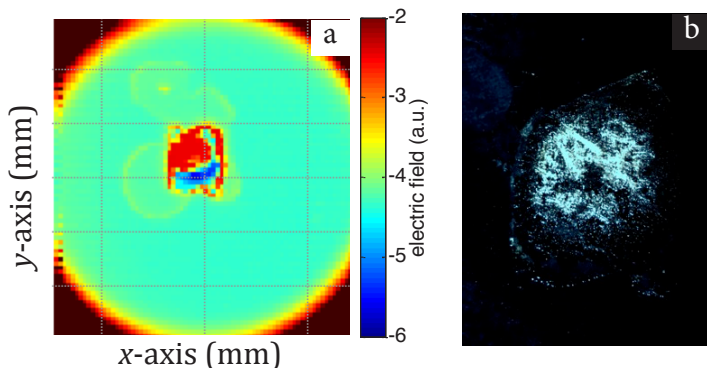


Figure 11. THz cross sections of 4T1 spheroids from 150,000 starting cells, second sample. The sample was embedded in agar gel and processed in its entirety using the THz spectrometer and imaged at 4× magnification. Figure a, shows positive reflection in the red area and negative reflection in the darkest blue area, corresponding to the area of highest density and area of low density in the microphotograph shown in Figure b. The image was taken of the entire sample in a petri dish.

Figure 12a also supported the previous findings. The red and black areas showed positive reflection, with black having a higher magnitude. The two black areas in the larger spheroid corresponded to the split spheroid seen in Figure 12b, although the orientation did not completely correspond. The second, smaller tumor was also seen in the microphotograph and measured 2mm in diameter while the larger one measured 5mm in diameter.

All THz images presented in this work represent the peak of the electric field reflected from the samples expressed in arbitrary units (a.u.), which could be the positive peak or the negative peak of the pulse.

Discussion

Using the round-bottom multiwell plate helped aggregate the 4T1 cells together and gave them greater opportunities for cell-to-cell contact to form the primary tumor as well as the peripheral spheroids. The sample obtained from the round-bottomed well was 25×the size of the largest spheroid grown in the flat-bottomed plates. However, the environment in the flat-bottomed plate ensured the tumor was fully encased in gel, as it would be in an actual patient. Growing in this environment is not an option for this project, though, because the THz spectroscopy was not able to produce results from the sample. The insignificant reflection of the electric field shown on the THz image is likely

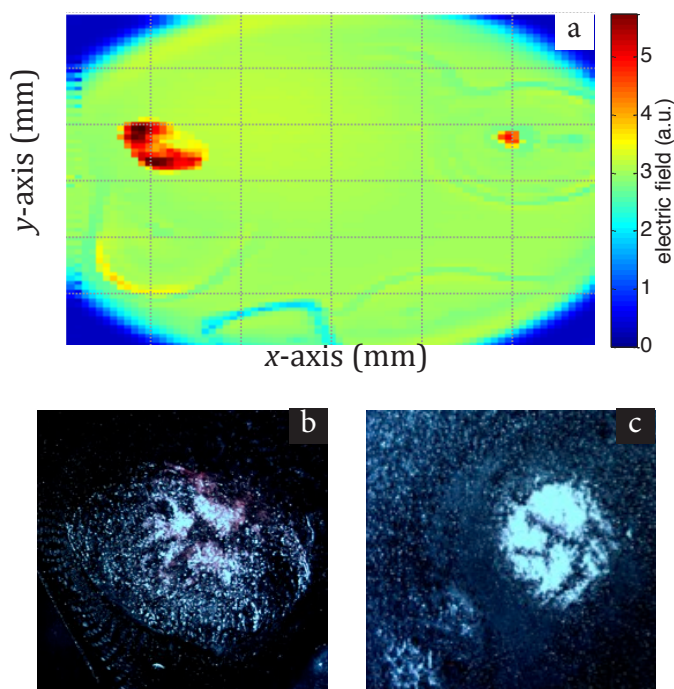


Figure 12. THz cross sections of 4T1 spheroids from 150,000 starting cells, third sample. The sample was embedded in agar gel and processed in its entirety using the THz spectrometer and imaged at 4× magnification. Figure a, shows positive reflection in the red areas and even stronger positive reflection in the black areas, corresponding to the multiple areas of thick cell density area in the microphotograph shown in Figures b and c. The image was taken of the entire sample in a petri dish.

due to the spheroids being too small for viewing, or the section could have been too thin leading to desiccation and loss of sample.

In the round-bottom environment, the uniform dispersal of cells starting with more than 150,000 cells suggests there is an upper limit to the cell count that can grow in a multiwell plate of this size. Potential causes are lack of space, lower nutrient:cell ratio, and limited access to the nutrients contained in the media. In order to remove the entire sample from the well and ensure all parts of the tumor were removed as a whole; freezing initially seemed to be the best option because it is also required for sectioning. However, the agar gel became too brittle at the -24°C temperature in which it is sectioned, and prevented a quality sample of the actual tumor from being included with the OCT Compound in the sample slice. This is shown in Figures 5-7 where the OCT produced electric field reflection different from that of the viewing plate, but the center of the plate had the same reflection as the plate. Additional samples were placed onto different material, polyurethane, to

ensure this was not the result of the sample having a refractive index equal to that of the original glass slide. Figure 8 also supports the concept that the wells cannot hold that many cells as it produces a uniform electric field reflection across the entire slide.

Transferring the sample directly to the viewing plate corrected this problem while still differentiating between the media and the 4T1 cells and eliminated the need for the OCT compound. The resolution of the THz system was not able to distinguish between the entire sample and the tighter spheroids that were attached to the outer edge of the tumor. This would be of less significance when dealing with a patient with breast cancer because the tumor would be a size large enough to be detected using the THz system.

Microphotographs of the spheroids from the 150,000 wells strengthen the findings of the THz imaging. Figure 10b aligns with 10a and shows a higher cell concentration and tumor height on the left of the image. More cells are seen on the right, with a much lower density in between. Figures 11a and 11b also correspond to each other, showing decreased tumor thickness on the bottom left corner of the sample. Finally, Figures 12b and 12c illustrate the results obtained in 12a. The microphotograph reveals the two dark areas within the tumor as areas of greater tumor thickness and gives more detail to the smaller, separated spheroid.

Based on the results, particularly Figure 12c that shows a separate smaller spheroid that grew in addition to the primary tumor, THz imaging and spectroscopy is a viable method for detecting breast cancer tissues of patients with tumors 2mm or larger. This size is much lower than the current median detectable size, 7.5mm (Michaelson et al., 2003). For future research, it is recommended plating 150,000 4T1 cells in a 96 well multiwell plate with a base layer of agar maintaining the rounded shape. To prepare and image the sample using the THz spectrometer, the sample should be transferred directly to the viewing plate without prior freezing and all water should be evaporated overnight.

The initial goal of this project was to grow breast tumors in vitro that mimic in vivo tumors in size and encasement in a flexible substance. While the tumors were limited in size, the results revealed a new methodology for growing tumors for THz spectroscopy at a lower cost that could be replicated on a larger scale.

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INTERFACE PROPERTY OF COLLAGEN AND HYDROXYAPATITE IN BONE AND DEVELOPING BIOINSPIRED MATERIALS

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Abstract

Bone at the nanoscale consists of type I collagen and hydroxyapatite (HAP). Type I collagen and HAP $[\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2]$ are responsible for most of the structural integrity of bone. Collagen fibrils contain HAP platelets of varying size dispersed between the collagen. We investigate heterotrimeric collagen interaction with HAP using Steered Molecular Dynamics to obtain the force-displacement relation as the collagen is undergoing shearing and peeling on the surface of HAP. Results indicate that the collagen requires 40% less force to separate from the HAP surface under peeling, when compared to shear loading. The Bell model is applied to determine the energy associated with key mechanisms under shear loading conditions. In both shearing and peeling, the number of collagen-water hydrogen bonds increases by approximately 100% before rupture. We developed an HAP inspired structure and 3D printed it using ABS plastic. This bio-inspired material could have several potential applications in engineering and medicine.

Introduction

Bone consists of a complex hierarchical system that on its own expresses astonishing characteristics of strength and flexibility. This biological hierarchy at the highest scale consists of a strong outer compact layer with a spongy interior. At near microscale, the compact bone is made up of osteons and Haversian canals (Launey, Buehler, & Ritchie, 2010). Inside the osteon system at the microscale there are multiple arrangements of fibers.

Expanding the scale down further shows the fibers from (d) in figure 1, made up of fibril arrays. These fibril arrays consist of a matrix of organic collagen and the mineral apatite (Launey et al., 2010). The Lamellae fibers (d) are made of small fibrils (f). These small fibrils are made up of an even smaller complex matrix of collagen and apatite (g). The bottom right panel (h) displays the interface between a segment of one tropocollagen molecule and the surface of HAP. HAP platelets range in size from 1 to 7 nm in thickness, 15 to 200 nm in length, and 10 to 80 nm in width (Fratzl, Gupta, Paschalis, & Roschger, 2004; Gupta et al., 2006; Ji & Gao, 2004; Weiner & Wagner, 1998). Collagen forms in lengths of about 300 nm and diameters of 1.5 nm (Launey et al., 2010).

It is important to understand the mechanical properties of the collagen-HAP interface at the nanoscale, because research shows how a change in mineral density at this level greatly affects the macroscale properties of bone (Nair, Gautieri, Chang, & Buehler, 2013). The goal of this study is to uncover whether or not different shearing and peeling loading conditions play a direct role in the mechanical properties of the

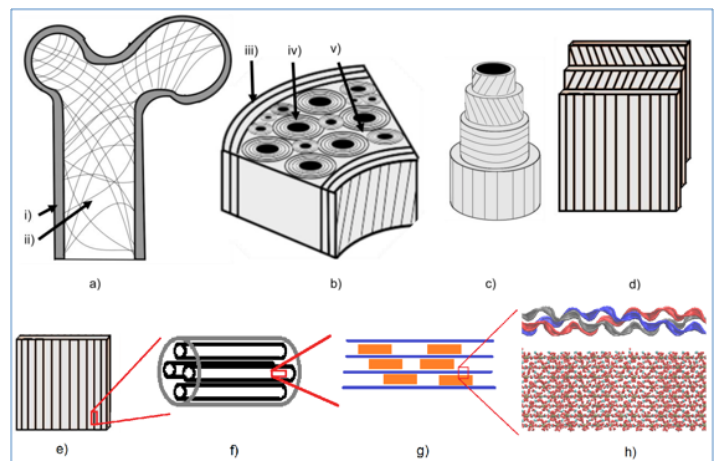


Figure 1. Bone Structure: a) Basic Structure ~15 cm i) Compact Bone ii) Spongy Bone b) Structure of Compact Bone ~5 cm iii) Circumferential Lamella iv) Osteon System v) Interstitial Lamella c) Osteon System ~ 100 μm d) Structure of Lamellae ~50 μm e) Single Lamellae f) Fibril Arrays ~10 μm g) Mineralized Collagen Fibrils ~1 μm h) Collagen-HAP Interface ~300 nm

interface. The scope of this research project does not include the process of forming collagen on the surface. By using Steered Molecular Dynamics (SMD) (Izrailev, 1998), we expect to learn more about the mechanical properties of solvated collagen along the surface of HAP crystals. SMD simulation provides force-displacement profiles for the system and the trajectory of the atoms simulated. The trajectory of atoms provides the data necessary to study the hydrogen bonds associated with the collagen-HAP system. The force and displacement can be used by the Bell model to predict the energy barrier for several mechanisms (Ackbarow & Buehler, 2009; Mirzaeifar, Qin, & Buehler, 2014; Qin, Cranford, Ackbarow, & Buehler, 2009). We hope for the results of this research to inspire the development of 3D printed materials based on the collagen-HAP system.

Theory

Tropocollagen Molecules

As stated earlier, collagen forms at lengths of about 300 nm in the collagen-HAP matrix (Launey et al., 2010). These long collagen molecules contain thousands of residues, which would be too computationally expensive to atomistically simulate in a full-scale collagen-HAP matrix. The collagen used in these simulations is modeled after real type I α -1 and type I α -2 chains of a common house mouse (*mus musculus*) and equilibrated in earlier work (Chang, Shefelbine, & Buehler, 2012; Libonati, Nair, Vergani, & Buehler, 2014; Rainey & Goh, 2004). The two chains were then reduced to 57 residues each to reduce computational costs. For this study, two α -1 and one α -2 chains are used to define the structure of the collagen as defined in (Gautieri, Vesentini, Redaelli, & Buehler, 2011). Ideal collagen consists of a Gly-Pro-Hyp triplet (Glycine-Proline-Hydroxyproline), but in nature the only reoccurring member of this triplet is Gly. The Pro and Hyp positions in the chains are often substituted by other common residues. The truncated chains hold the same composition as the full length chains. Full information about the construction of this molecule can be found in the work by (Chang et al., 2012)).

For the simulations considered in this study, the protein data bank (PDB) files associated with the heterotrimeric molecule is completed by creating compatible protein structure files (PSF) for each molecule using Visual Molecular Dynamics (VMD)

(Humphrey, Dalke, & Schulten, 1996). The N-terminus of each chain is capped with an acetyl group (ACE), while the C-terminus is capped with trimethylamine (CT3) to produce charge neutrality across the entire chain.

Hydroxyapatite Crystal

The hydroxyapatite mineral used in this research is made up of hexagonal unit cells containing 44 atoms with the lattice parameters: $a=9.4241\text{\AA}$, $b=9.4241\text{\AA}$, $c=6.8814\text{\AA}$, $\alpha=90^\circ$, $\beta=90^\circ$, and $\gamma=120^\circ$ (Libonati, Nair, Vergani, & Buehler, 2013). These unit cells are then replicated 24 times along the a-axis, 4 times along the b-axis, and 4 times along the c-axis. The collagen is placed on the OH surface of the HAP because of its negative charge. This allows more hydrogen bonds to form via the increased donors and receptors (Qin, Gautieri, Nair, Inbar, & Buehler, 2012).

Force Fields

This system is modeled using a modified CHARMM force field originally found in reference (Qin et al., 2012), because it has been tested to simulate collagen-HAP systems as demonstrated in the work by Qin and colleagues (2012) and Libonati and colleagues (2014). Included in the force field parameters are quantum mechanical calculations derived for hydroxyproline (Hyp), which is found in collagen, but not in other proteins (Park, Radmer, Klein, & Pande, 2005). Nonbonded parameters were originally calculated using a Born-Mayer-Huggins model (Bhowmik, Katti, & Katti, 2007; Hauptmann, Dufner, Brickmann, Kast, & Berry, 2003). This force field has been validated in previous works, (Bhowmik et al., 2007; Dubey & Tomar, 2009; Hauptmann et al., 2003; Qin et al., 2012; Shen, Wu, Wang, & Pan, 2008).

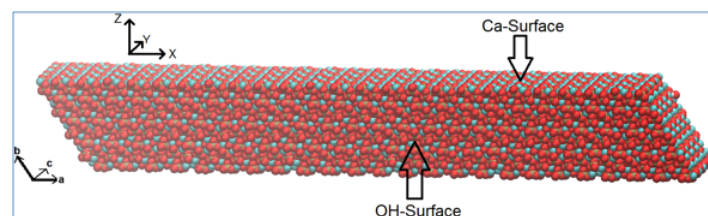


Figure 2. Diagram of HAP Crystal. Surfaces are labeled accordingly. The a, b, and c axes are based on the HAP lattice parameters while the Cartesian coordinates are associated with the simulated system.

Because this system equilibrates with an unsolvated collagen and later with a solvated collagen, a modified CHARMM force field is used. The force field for the unsolvated collagen $\frac{1}{r^2}$ (stated above) has a Coulombic energy that varies with $\frac{1}{r}$ and is thus a dielectric term that varies with $\frac{1}{r}$ distance. When water is added to the system, $\frac{1}{r}$ explicit water molecules must be accounted for in the Coulombic energy by taking away one of the $\frac{1}{r^2}$ terms to have the energy drop off linearly with distance (Plimpton, 1995). This change is important in simulating solvated biomolecules. Throughout each simulation, Lennard-Jones and Coulombic interactions are calculated by the modified CHARMM fields by ramping energy and force smoothly to zero between the inner and outer cutoff regions of 8Å and 10Å. This has been validated in previous research (Libonati et al., 2014; Plimpton, 1995).

Bell Model

The Bell model is a theoretical model that combines known parameters of certain bonds and angles with the results of a Molecular Dynamics (MD) simulation to calculate the energy barriers of said bonds (Ackbarow & Buehler, 2009; Mirzaeifar et al., 2014; Qin et al., 2009). In this study we apply the Bell model to the rupturing of the hydrogen bonds. This allows us to relate a force-displacement to the energy associated with a mechanism. For example, this can be applied during an MD simulation where multiple bonds are broken and new states of equilibrium are reached. The equation for the Bell model used in this study is given (Mirzaeifar et al., 2014) as

$$F = \frac{k_b T}{x_b} \ln \left(\frac{V}{\omega_l} \right) + \frac{E_{b1}}{x_b} \quad (1)$$

where, F is the force during rupture, k_b is the Boltzmann constant, T is the absolute temperature of the system, x_b is the slip distance, V is the loading rate, ω_l is the natural frequency of a hydrogen bond, and E_{b1} is the energy barrier of the bonds. Without the Bell model, these energy barriers cannot be determined, as the only variable is force, but knowing the temperature, natural frequency of bonds, and pulling velocity allows us to apply the Bell model to estimate the energy barriers associated with each mechanism.

Methods

Unsolvated Equilibrium

All simulations are conducted using LAMMPS (Plimpton, 1995) with a 1.0 fs timestep. This system is minimized by LAMMPS using two different methods. The first is the conjugate gradient while the second is the steepest descent method. After this minimization process, the system is equilibrated under an NVE ensemble using a Langevin thermostat over 125 ps ramping the temperature from 250 K to 300 K. The bottom single layer of calcium phosphate in HAP is held fixed, while the system is equilibrated again at 300 K for 50 ps. Equilibrium is confirmed by checking the Root Mean Square Distance (RMSD) using VMD (Humphrey et al., 1996). The final frame of the unsolvated system is used as the starting frame for the solvated system.

Figure 3 shows a diagram of the unsolvated system. The HAP is positioned so that the Ca-rich surface is normal to the z-axis, while the OH-rich surface is normal to the y-axis. As shown in the top-right of figure 3, “Peeling” in this report refers to pulling along the y-axis, while “Shearing” refers to pulling along the x-axis.

Solvated Equilibrium

After the equilibration of the unsolvated system, a box of water molecules using the TIP3 model is added using VMD (Humphrey et al., 1996) around the collagen and extended in the predicted trajectory of the collagen during SMD simulations. For the system where shearing is tested, water is added in a 41 x 5.5 x 3.0 nm³ box extending 23 nm in front of the predicted shearing trajectory. Along with this 23 nm lead, at least 0.8 nm of water is placed around the collagen to ensure consistent solvation.

As shown in figure 4, more than the entire length of the collagen was predicted to uncoil and shear off the surface in this test. The system setup for peeling

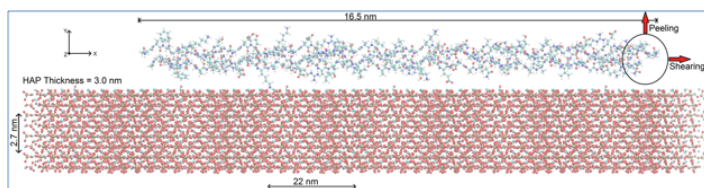


Figure 3. Diagram of the Collagen-HAP System

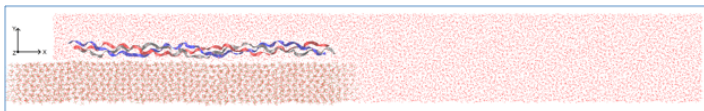


Figure 4. Box of water added to system prepared for shearing

is solvated using the same method as above, except with a box of water extended along the y-axis to ensure full solvation during the simulation. The box of water added for the peeling scenario extends 11 nm above the top of the equilibrated collagen as shown in figure 5.

As illustrated in figure 5, water is added to give the collagen enough room to separate from the HAP considerably before escaping the water box, but not enough water is added to maintain full solvation for full separation. This decision was made to reduce the computational time required to simulate the system. Both of the systems each contain about 75,000 atoms and require simulation times on the order of weeks using 12 parallel 16-core processors.

As stated above, both systems are minimized and equilibrated using the same parameters as before with the only difference being a slight change in the modified Coulombic force field. Once explicit water has been added to the system, the Coulombic energy equation is modified to vary linearly with distance as opposed to an inverse squared relation for the unsolvated collagen. After the solvated system has been verified at an equilibrium state (once again checked using RMSD) the system is setup to run under SMD (Izrailev, 1998) calculations.

Steered Molecular Dynamics

Steered Molecular Dynamics (SMD) works by

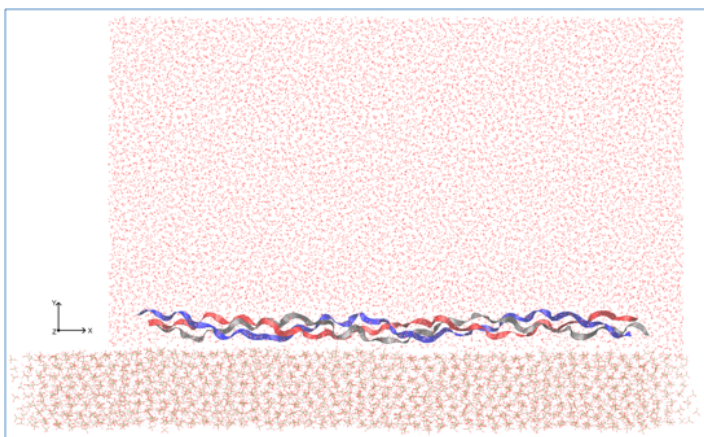


Figure 5. Box of water added to system prepared for peeling

tethering a virtual spring to an atom or group of atoms and pulling the other end of the spring at a constant velocity (or constant force, not used here) (Izrailev, 1998). Here, one end of the spring is tethered to the final C_{α} atom of each chain. By tethering the spring to three identical atoms, SMD effectively normalizes the masses of the atoms and sets the tethered point to the center of mass of the atoms. The untethered end of the spring is positioned at the same location as the center of mass to ensure zero initial force. Then, depending on whether or not we want to shear or peel the collagen we set the untethered end of the spring to pull at a constant velocity along that axis. The velocity parameter picked here is set to $0.00001 \frac{\text{\AA}}{fs}$ or $(1 \frac{m}{s})$. This velocity has been tested on collagen-HAP systems and validated in previous works (Gautieri, Uzel, Vesentini, Redaelli, & Buehler, 2009; Libonati et al., 2014; Qin et al., 2012). The spring constant is set to $10 \frac{kcal}{mol \cdot \text{\AA}^2}$ or $(\sim 6.95 \frac{N}{m})$ et al., 2009; Libonati et al., 2014; Qin et al., 2012).

Post-Processing

All systems are visualized using VMD (Humphrey et al., 1996) along with calculating RMSD, solvating the systems, creating PSF files, and computing hydrogen bonds. Calculating hydrogen bonds using VMD requires a cutoff-distance and cutoff-angle. As previously used successfully in (Libonati et al., 2014; Nair et al., 2013; Qin et al., 2012), these parameters are set to 3.5 Å and 30° respectively. MATLAB is used to process results and produce plots for force vs. displacement profiles and hydrogen bond vs. displacement profiles. The displacement distance used in all calculations is the displacement of the untethered end of SMD's virtual spring.

The Bell model is applied using the force vs. displacement plots and known parameters about hydrogen bonds.

k_B	$1.380 \times 10^{-23} \frac{J}{K}$
T	300K
V	$1 \frac{m}{s}$
ω_1	$1 \times 10^{13} Hz$

Table 1: Constants k_B , T , V , and ω_1 used in the Bell Model

Table 1 lists the constants and their units used in the Bell model. Once the energy barrier is calculated, it is converted into atomistic statistical units of $\frac{kcal}{mol}$. The force and rupture length are determined from the SMD simulations.

Results and Discussion

Shearing and Peeling of Heterotrimeric Collagen-HAP

We simulate the shearing system using SMD for approximately 57 ns. During this time the force reaches 330 nN before the collagen uncoils. The peeling system is simulated for 42 ns with a maximum force of 205 nN. This is shown in figure 6. It should be noted that maximum forces obtained here are several orders of magnitude lower than the strength of bone at the macroscale (Evans & Lissner, 1957).

Figure 6 compares the force vs. displacement for the shearing and peeling systems. As can immediately be seen, collagen has a much lower resistance to being peeled from the surface as opposed to shearing across the surface. Peeling the collagen from the surface induces rupture well before shearing the collagen at about 40% less force. Peeling collagen starts with a similar force-displacement trajectory, but gradually diverges from the shearing system. Figure 6 shows how the stiffness of the peeling system decreases at a faster rate than the shearing system as the collagen separates.

Work done by Tao and colleagues (2015) relates the rupture force of collagen to the surface of HAP using loading rate as a variable. Their experiments show that

the forces required to rupture collagen from the surface of HAP increases with faster loading rates, but there is also uncertainty involved. The loading rates used in our study are many orders of magnitude higher than the data shown by Tao et al. (2015), and the rupture forces calculated are also many orders of magnitude higher than the experiment. Although not directly comparable to each other, the forces at least appear to follow the trend shown by Tao et al. (2015).

Force-displacement plots by themselves do not capture the full picture in explaining the similarities and differences between how collagen interacts with itself and the surface of HAP. Hydrogen bonds play an important role throughout the SMD simulations.

Figure 7 shows the plot of collagen-collagen hydrogen bonds formed during the SMD simulation. It is interesting to note that peeling collagen consistently forms about 15% fewer bonds with itself, compared to shearing collagen, until the peeling collagen ruptures from the surface. This shows that the loading conditions applied to the collagen reduce the probability of forming hydrogen bonds within itself.

Figure 8 displays the plot of hydrogen bonds formed between the collagen and HAP during the SMD simulation. The bonds formed between the collagen and HAP for peeling collagen start about 30% lower than shearing collagen with this gap increasing up to nearly 50% after 100 Å displacement.

Figure 9 shows the hydrogen bonds formed between the collagen and water as the collagen is simulated using SMD. As expected, as both collagen

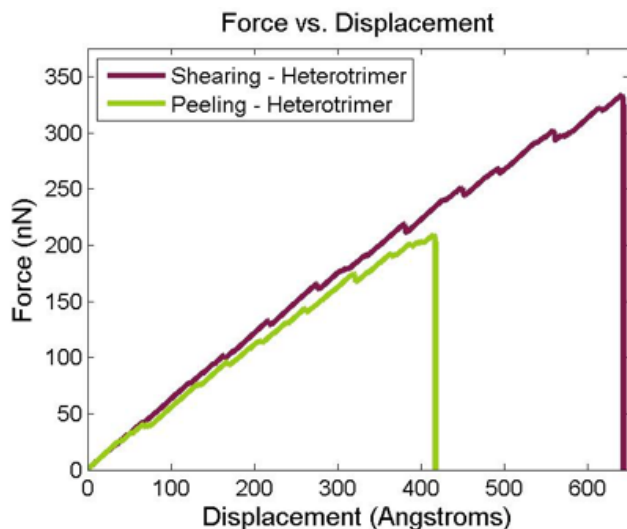


Figure 6. Force vs. Displacement of Heterotrimeric Collagen for Shearing and Peeling Loading Conditions

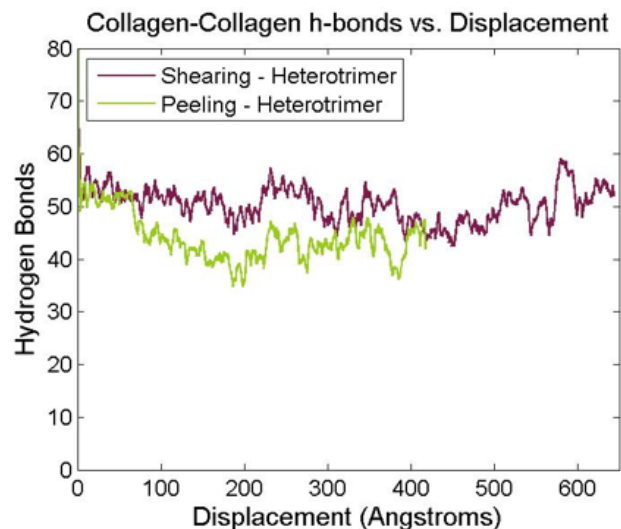


Figure 7. Collagen-Collagen Hydrogen Bonds vs. Displacement for Shearing and Peeling Loading Conditions

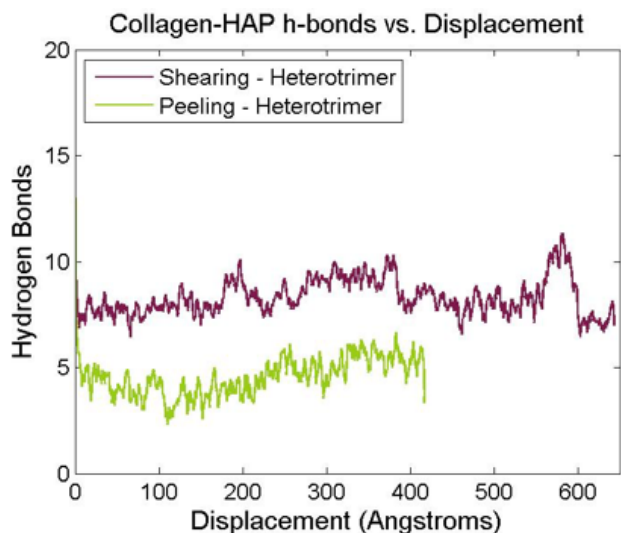


Figure 8. Collagen-HAP Hydrogen Bonds vs. Displacement for Shearing and Peeling Loading Conditions

molecules start uncoiling they start forming more bonds with the water. This is caused by an effective increase in surface area of the collagen through exposed atoms.

Applying Bell Model to Shearing System

To apply the Bell model to this system, it is important to examine locations where the collagen has multiple hydrogen bond ruptures and slips. The most interesting locations for this include the first slip of the simulation and a large slip in the final stages. These slips are characterized by the collagen moving along the surface of HAP.

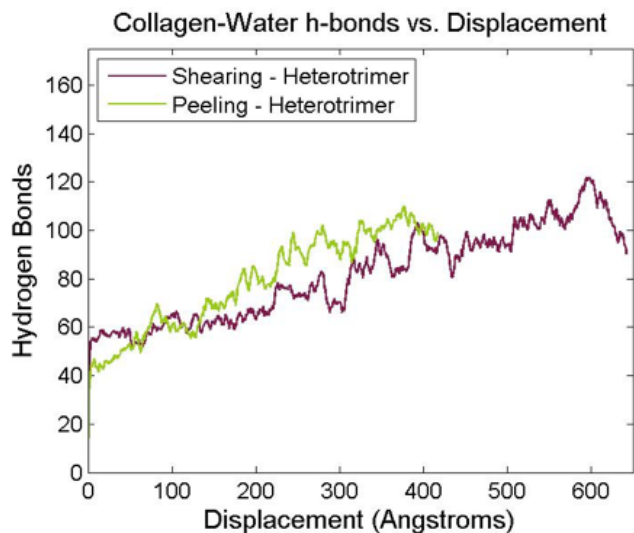


Figure 9. Collagen-Water Hydrogen Bonds vs. Displacement for Shearing and Peeling Loading Conditions

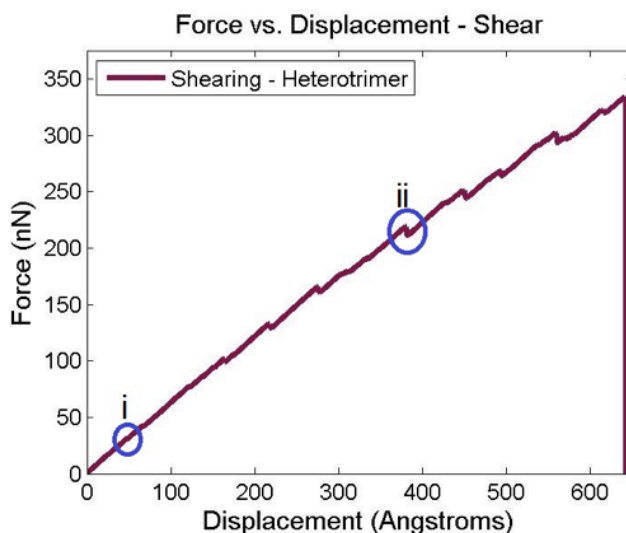


Figure 10. Locations of First and Final Slips: i) First slip for shearing, ii) Final slip for shearing

Figure 10 shows the locations of the first and final slips of the shearing system. The blue circles represent the locations of collagen slips. A careful examination is conducted for each location.

As seen in figure 11, the final slip creates a greater drop in force than the first slip. This is because the forces at lower displacements are enough to break bonds, but not strong enough to create huge slips of the collagen. In the top left panel (i) the rupture force, F , is 31.25 nN at a rupture distance, x_b , of 1 Å while the top right panel (ii) has a rupture force of 219 nN over a distance of 2 Å. The lower left panels show the extent of the first slip in the shearing system.

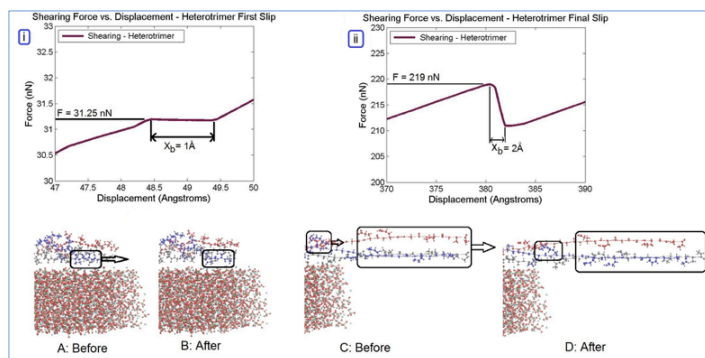


Figure 11. Zoomed-In Heterotrimeric Slip Force vs. Displacement: i) Location of first slip, ii) Location of final slip, A) Image from before first slip, B) Image from after first slip, C) Image from before final slip, D) Image from after final slip.

We apply the Bell model to the locations highlighted previously and calculate the following energy barriers for shearing collagen.

As illustrated in table 2, the energy barrier is considerably increased for slips that occur at higher forces. The number of hydrogen bonds associated with this much energy must come from the water-water hydrogen bonds breaking, because there are not enough bonds formed to the collagen itself to produce this much energy. This could occur due to the static nature of hydrogen bonds. The system never moves at a steady rate; it can only form, break, and reform bonds to reach new points of equilibrium.

Location	Energy Barrier ($\frac{kcal}{mol}$)
i: First Slip for Shear	468 kcal/mol
ii: Final Slip for Shear	6320 kcal/mol

Table 2. Energy barriers associated with the first and final slips in the shearing collagen system

Biologically Inspired Materials Based on Collagen-HAP System

Modern advances in engineering materials research are strongly based on biological materials. Nature has had billions of years to evolve different biological structures. This is especially true in the case of the structure of bone. Bone is the most complex hierarchical structure found in nature, and it is important to study these complex natural systems in order to produce synthetic materials with custom properties. For example, before powered flight was made possible by the Wright Brothers in 1903, many engineers looked to birds for inspiration. Early concepts of flying machines strongly resembled the flapping motion of birds' wings. More recently, scientists and researchers examine biological materials at the nanoscale to further understand how the nanoscale structure of materials contributes to the overall macroscale mechanics. For example, carbon nanotubes consist of rolled sheets of graphene to create tubes of carbon atoms that can potentially be weaved together like a braided rope to create materials that are much stronger than steel. The research conducted here has inspired us to

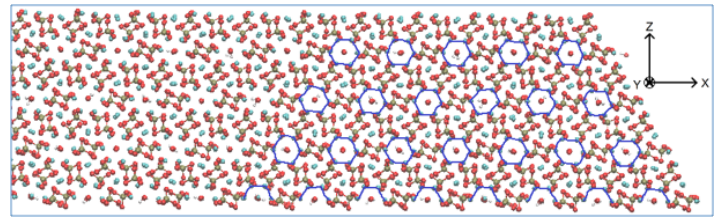


Figure 12. HAP OH-Surface Highlighted to show hexagonal channels

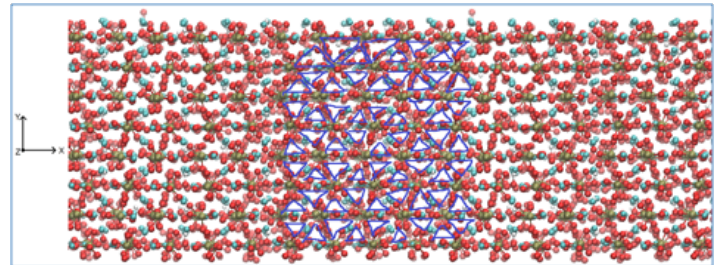


Figure 13. HAP Ca-Surface Highlighted to show triangular channels

design a 3D model of the collagen-HAP system and print it using an ABS plastic additive printer. When conducting simulations on the system we noticed the geometric shapes that the OH channels form along with the smaller channels formed through the Ca surface. Below is a labeled snapshot highlighting the hexagonal OH channels.

Figure 12 depicts how the OH channels take the shape of hexagons through the HAP cells. These channels run completely through the HAP in the face. The blue hexagons drawn highlight the borders of these channels. The calcium atoms form the corners of the channel.

Figure 13 shows a snapshot of the Ca-surface of HAP with the triangular channels highlighted using blue triangles. These channels are particularly interesting, because the position and orientation of the triangles appear to be broken down hexagons. This shows that HAP crystal must obtain its strength and rigidity through these geometric organizations. We combined the two observations to produce the model shown in figure 14.

Figure 14 displays the final design of the bio-inspired 3D model. The top surface contains multiple hexagons that represent the OH channels seen in real HAP crystals.

The structure of the hydroxyapatite crystals studied here could lead to the development of multifunctional macro-materials based on the three-dimensional hexagonal structure paired with triangular

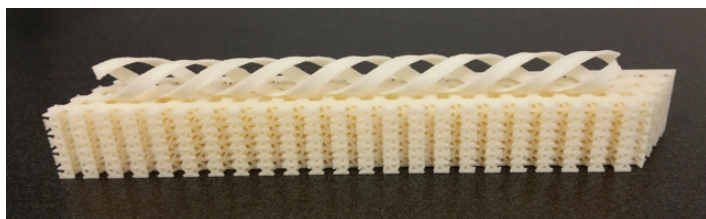


Figure 14. 3D Printed Collagen-HAP System

channels. Perhaps professional athletes who suffer from bone fractures or broken bones could have HAP-inspired implants that could promote rapid bone growth and allow the athlete to recover much faster. Much like baking a perfect soufflé, creating revolutionary new materials requires more than one ingredient. HAP could potentially be paired with two-dimensional layers of graphene to produce stronger biological tissues. Graphene-coated HAP could lead to completely new types of biological tissues with vastly different properties to the purely biological counterparts. In the Marvel comics, the fictional James Howlett (also known as “Wolverine”) had his entire skeleton laced in a mysterious fictional super-alloy known as adamantium (Claremont, 1976). Perhaps the first real-world counterpart to this fictional hero will have his/her bones laced in graphene-coated HAP instead of this mysterious super-alloy.

Conclusions

We use SMD simulations to study the mechanisms associated with heterotrimeric collagen, when interacting with HAP under shear and peeling loading conditions. We use the Bell model to predict the energy barrier of several mechanisms under shear loading. Heterotrimeric collagen under shearing loading conditions is much more resistant to failure than peeling loading conditions. Peeling collagen results in a 40% reduction in the force required to rupture the collagen from the surface when compared to shear.

Analyzing the shearing system with the Bell model predicts the energy barriers associated with key mechanisms (e.g. collagen uncoiling) of heterotrimeric collagen under shear force. The energy barriers calculated using this model could be used for macroscale models of bone to define failure criteria.

Materials and objects can be designed based on the nanostructure of HAP. Geometrically optimized channels in HAP led to the development of a macroscale

3D printed model of the entire system.

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