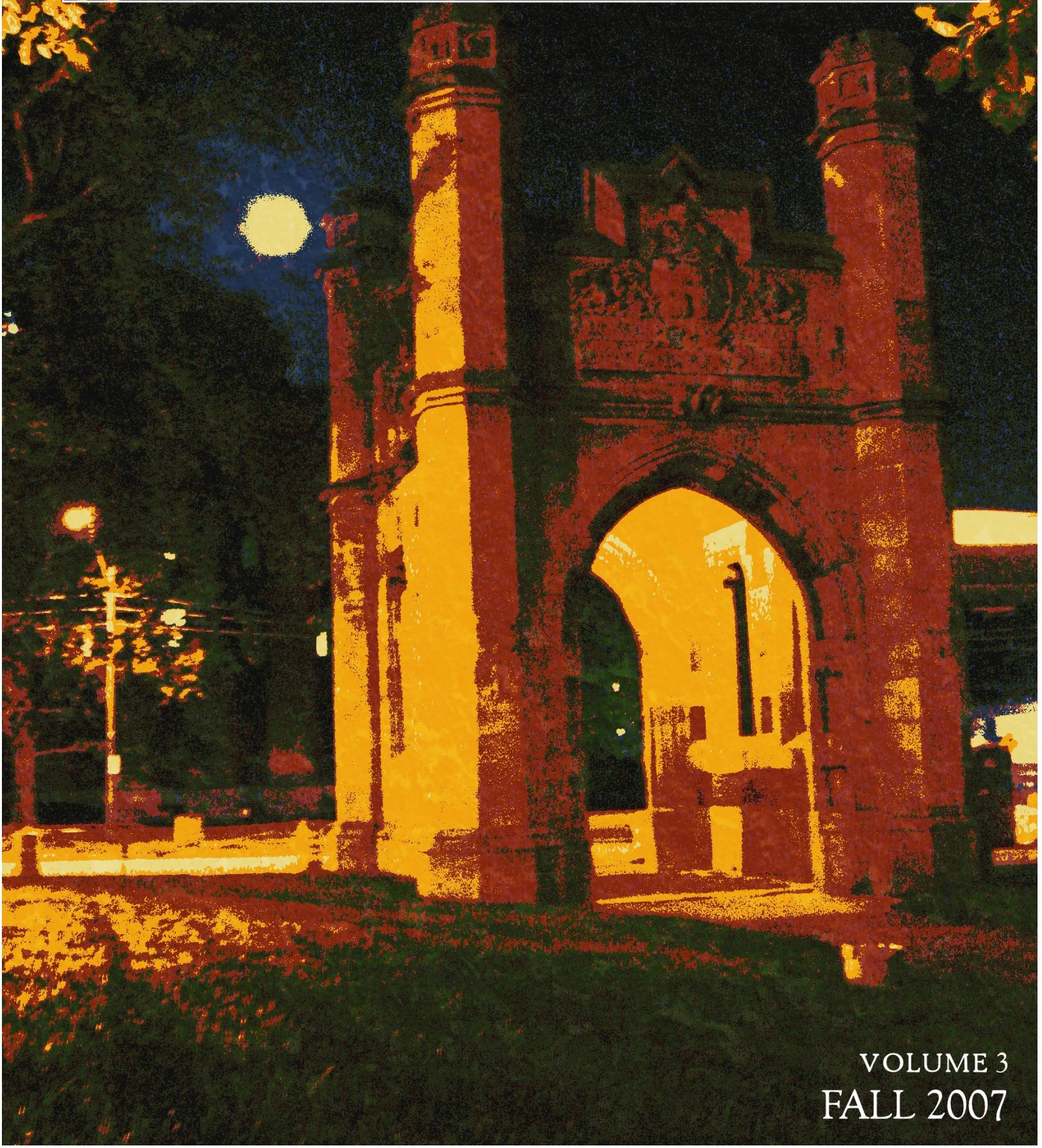
DISCUSSIONS

CASE WESTERN RESERVE UNIVERSITY
UNDERGRADUATE RESEARCH JOURNAL



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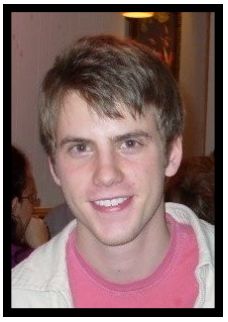

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Letter from the Editors

The 2007-2008 academic year brought many changes for *Discussions*. One of our most important changes was increasing publication to two issues per year. Doubling our number of issues required us to reevaluate both our review system and time allocation. At multiple points during the semester it seemed as if our efforts at putting together a fall issue as well as a spring issue were going to collapse on top of us. However, the combined resolve of our officers and reviewers paid off, producing here a volume that, while assembled in an interval of half the time, retains all the quality that previously took a year to compile.

Another change came with the graduation of *Discussions'* founding officers in the spring of 2007. Fortunately, this year's Editorial Board met the challenge with a keen enthusiasm and an innovative spirit. This energy allowed us build upon the foundation established by the original members.

One of the more innovative ideas brought in this year is publishing the journal in CD format as well as in printed form. We hope that this alternative method of publication will not only appeal to the technophiles of our audience, but also allow for a more compact and convenient means of distribution. Keep an eye out for both forms of publication, and grab your copy of choice!

We would like to thank SOURCE and Media Board for their continued support. Media Board's support allows us to publish the journal and explore new ideas, such as the aforementioned CD production and a new, more professional binding. Our advisors Sheila Pedigo and Bethany Pope of SOURCE are endless sources of guidance for us, and their constant readiness to help us in any way possible is very much appreciated.

The articles included in this issue span a variety of disciplines, and we hope that all readers and reviewers are able to find something of interest within our pages. As always, we encourage everyone to submit their own research for future publications. We accept research from all disciplines--whether you study biomedical engineering or art history, we'd appreciate your essays. The submission deadline for our Spring 2008 issue is February 3, and submission guidelines can be found on our website at www.case.edu/source/discussions.

We also welcome any students interested in becoming reviewers for *Discussions*. As a student run organization, we rely completely on participation from the undergraduate student population at CWRU! The only skills or previous experience necessary to becoming a member of *Discussions* is the ability to read and form constructive criticism of the papers we review. Join us!

Thank you for picking up the Fall 2007 issue of *Discussions*, and we hope you enjoy it.

Anna Wieser and Sean Yeldell
Editor-in-Chief and Managing Editor

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Discourse in Programming: Chafe Applied to Computer Code

-Steven Dee-

Steven is currently in the second year of a double major in computer science and cognitive science here at Case. Prior to coming here, he did some post-secondary coursework at the University of Akron, He's pledged Theta Chi and has also been involved in CWRU Film Society, the ACM, and the math club. Steven's current plans are to start up a recreational computer science and programming club on campus.

-Acknowledgements-

I'd like to thank Todd Oakley for his assistance in writing the paper, as well as for encouraging me to submit it for publication; Discussions, for electing to publish it; the cognitive science department here for providing a platform for this sort of research; Dr. Kathy Liszka at Akron for first encouraging me to branch out into alternative computer languages; Dr. Jeantet for introducing me to the field of linguistics; and Dr. Ulm for reminding me that as a computer scientist, I must decide whether to be a good wizard or a bad wizard.

Discourse, at its absolute broadest definition, can be seen quite simply as the exchange of information. In its most common occurrence, this information is human thought exchanged between participants in conversation; however, the participants need not be constrained exclusively to humans. What a programmer, for instance, does in writing code can be viewed very easily as holding discourse with a computer. Indeed, it can be very instructive to analyze programming from a discursive perspective; deficiencies in programming languages can be revealed by their deviation from conversational rules, and the nature of all discourse and cognition can be explored with an eye to phenomena that occur in programming. An analysis with regard to the linguistic framework Wallace Chafe presents in Discourse, Consciousness, and Time can be particularly enlightening in this respect.

Chafe, a professor of linguistics at the University of California Santa Barbara, has analyzed human speech with special attention to its prosodic features—its flow, speed, tone, et cetera. From this analysis, he has produced a means of reasoning about human speech production. Using this framework, it is possible to draw conjectures about language, and perhaps also about the nature of human thought.

In order to analyze programming languages with respect to Chafe's framework, we first need some heuristics. Chafe analyzes language primarily with respect to intonation units, but also with respect to subject/verb/object constructions and identifiers. An application of Chafe's framework to programming languages requires at least that we find analogous constructions in these languages.

In natural language, sentences can be broken up into a subject, a verb (or verb phrase), and an object. In programming languages, it is

useful to chunk statements in a similar way; in Java, for instance, the statement "`subject.action (object)`" can be read as "subject performs action on object." In some languages, though, actions are not tied to subjects in that manner. In C or Lua, for instance, one frequently sees constructions such as "`action (subject)`" or "`action(subject, object)`", and in Smalltalk, statements are of the form "`Subject messageVerb preposition: object`". In general, this paper and the accompanying examples will use the above constructions except when others are useful to illustrate a point.

Intonation units are of course a phenomenon in spoken language, which is rather far-removed from program source code. Source is not, however, devoid of analogous constructs. Lines of code in programming languages exhibit many traits of intonation units—they typically, for instance, obey (with some notable exceptions) Chafe's one-new-idea constraint. This is a limited heuristic—it does not account for the role of control structures or for the existence of whitespace-insensitive languages—but it is useful enough to be used throughout this preliminary survey.

Chafe observes the presence of a one-new-idea constraint (109) in intonation units. In programming, there exists an adage: "do one thing" (Atwood). That is, a function or variable should serve only one purpose. Generally referred to simply as common wisdom, this adage can be explained as a means of enforcing the one-new-idea constraint. It might be that a programmer has an easier time chunking and comprehending code that fits with his expectations for the contents of intonation units.

In his discussion of activation cost, Chafe separates information in conversation into the categories *given*, *accessible*, and *new* (74). In Chafe's framework, these categories correspond to speakers' and listeners' mental states during conversation. In this analysis, there are two components to this concept of accessibility: the programmer's mental state, and the information accessible to the computer during compilation or execution of the program. The programmer's perspective is rather loosely defined; when looking at or working on certain parts of a program, information relevant to those parts is likely to be given or accessible to the programmer.

From the computer's perspective, on the other hand, things are more openly defined. A non-trivial program frequently makes use of hundreds or thousands of variables throughout its source, and modern computers have the ability to hold effectively all of these in main memory. However, many of these are relevant only to one particular part of the program; they may be accessible to the computer, but not to the programmer, and their reference can cause confusion when reading source code. A problem in programming language design, then, is to provide as much correlation as possible between knowledge that is given or accessible to the program and knowledge that is given or accessible to the programmer.

At the low end of the spectrum is C, which provides only a bare minimum of scoping functionality. It is possible to refer to global variables from anywhere in the program code, and no means is provided of limiting access. This makes it possible for the program to reference information that is unknown to the programmer.

Programmers have worked around the deficiencies in C scoping in a number of ways. One common

practice is to prefix variables relevant to only one portion of the program with a particular string (usually an underscore), so, for instance, `_aCounter` refers to a "private" variable that should not be used elsewhere.

Another practice, demonstrated here within the Lua programming language, is to provide "closures" (Ierusalimschy §6.1). A closure is a block of code that can contain information both local to itself and inaccessible to the rest of the program. In this manner, a programmer will only see references to information that is given (previously defined within a closure) or accessible (relevant to the larger program) at any given point. For instance, on appendix A line 2, a variable ("`count`") is defined that is local to the closure. For the duration of code through which it is relevant (i.e., the closure), it can be referred to. In another closure, though, it cannot be accessed (and attempting to do so would raise an error).

Chafe indicates the existence of a light subject constraint. That is, subjects in conversational language are always either low-cost (given or accessible) or trivial (92). This constraint is enforced to some degree in most programming languages, but particularly well in object-oriented languages like Smalltalk or Java.

In Smalltalk, for instance, if one wanted to work with an instance of the `Person` object, one would first have to declare an instance variable: "`Jim := Person new.`" This can be read as "I declare `Jim` to be an arbitrary new `Person`." Here, `Jim` best fills the grammatical role of object rather than subject. Further, now that `Jim` has been declared (i.e., is *given* in the source code "conversation"), it can take on the role of subject: one can ask questions of it (e.g., "`Jim isHungry?`"), tell it to do things (e.g., "`Jim eat: aBurger`"), or (to use another example), manipulate it: "`3.14 truncated negated`", for instance, evaluates to -3 (Sharp 6). Smalltalk thus enforces the light subject constraint.

To a certain extent, the light subject constraint is satisfied by most programming languages in that variables must be declared (or at minimum, used in function arguments) before they can be used in a "subject" role. The complicating factor is, as discussed earlier, the discrepancy between accessibility of information to a program and to its programmers.

A common practice among experienced programmers is that of "playing computer." That is, an experienced programmer will frequently take on the computer's role in the "discourse" of programming, trying to determine the computer's internal state and actions at each step of execution of a program. Stepping back, it is informative to note the roles of the "participants" in this discourse: it is the programmer's goal to translate thought into code, and the computer's goal to translate code into computation and output. Yet, in performing his role, the programmer must continuously consider the role of the computer. In order for the discourse to succeed, the computer must interpret the programmer's code as intended; for this to occur, the programmer must know how the computer will interpret his code.

One particularly interesting potential area of study is the relation that this "playing computer" has to what we do in discourse with other people. It is currently an open question, for instance, whether activation cost is a reflection of delay incurred by the speaker in accessing information or an anticipation of delay incurred by the listener in accepting information. I submit that we may (albeit on a more natural, unconscious level) undergo a similar process in conversation to that

of the programmer in computing—that is, that in our discourse with other people, we unconsciously devote some energy to "playing human" in anticipating our listeners' role in conversation.

Chafe's framework appears particularly well-suited to studying the art of programming. Programming languages seem to adhere in many respects to the constraints he sets forth, and where they don't, problems and workarounds can be seen to arise. Moreover, the techniques used by programmers in this particularly specialized form of discourse can be instructive in the analysis of more general conversation. This preliminary analysis only scratches the surface; there are many further avenues of study available.

NOTES

1 Java is a popular object-oriented programming language, used frequently in computer science curricula as well as in corpo rate environments.

2 The C programming language, dating back to the 1970s, is one of the oldest computer languages still in wide use today. It is frequently used for low-level systems programming.

3 The Lua scripting language is commonly embedded within other programs as a means for users to extend them; it was de signed to be easy to use, even by people unfamiliar with it.

4 Smalltalk is a "pure" object-oriented language, whose goals are to be consistent and (as the name suggests) small—in fact, it first arose out of a challenge to fit an entire language specification on a single sheet of A4 paper.

5 In fact, there is a little more to C scoping than discussed here—in particular, static variables and per-file visibility can be used to reasonable effect in situations like the one shown. The example given here, though, while contrived, is indicative of real-world programming practice.

6 Alternatively, it could be stated that `Jim` is here new but trivial, or that this construction is in fact a violation of the light-subject constraint. Indeed, object declaration is one possible avenue of further exploration; however, the given explanation suffices for this survey.

RESOURCES

Atwood, Jeff. "Curly's Law: Do One Thing." Coding Horror. 01 March 2007. 9 April 2007 <http://www.codinghorror.com/blog/archives/000805.html>.

Chafe, Wallace. Discourse, Consciousness, and Time. Chicago: The University of Chicago Press, 1994.

Ierusalimschy, Roberto. Programming in Lua. Lua.org, 2003. 9 April 2007 <http://www.lua.org/pil/>.

Sharp, Alec. Smalltalk by Example: The Developer's Guide. Berne: The University of Berne, 1997. 9 April 2007 <http://www.iam.unibe.ch/~ducasse/FreeBooks/ ByExample/SmalltalkByExampleNewRelease.pdf>.

Serotonin-Dopamine Interactions in *Pet*-1 Knockout Mice

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ABSTRACT

Serotonin (5-HT) has been implicated in playing a role in the release of dopamine (DA) in the prefrontal cortex of the brain. Interactions between these two neurochemicals are believed to play a role in the regulation of brain functions such as memory and emotion. *Pet*-1 knockout (KO) mice are transgenic mice lacking a transcription factor that specifically regulates the development of brain 5-HT systems. As a consequence, most forebrain 5-HT neurons are not present in these mice. They will be used in this study to assess the effect of 5-HT depletion on the release of DA. The variable that will be measured is DA release in the prefrontal cortex in *Pet*-1 KO mice and wild-type controls. DA levels will be compared both before and after exposure to a mild psychological stressor. It is hypothesized that basal and stress-induced DA release will be diminished in KO mice. *In vivo* microdialysis will be used to collect extracellular DA in the mouse prefrontal cortex. In this technique, a microdialysis probe, which is perfused with a physiological solution and consists of a semipermeable membrane across which DA and 5-HT can pass, will be implanted into the mouse brain during surgery. The next day, the microdialysis probe will be perfused at a rate of 1.5 µl/min and dialysate samples will be collected every 20 minutes. The stressor (gentle handling) will be applied for 20 min once DA levels are stable. High Performance Liquid Chromatography (HPLC) with electrochemical detection will be used to measure the amount of DA in the dialysate samples. In HPLC, the fluids collected from the probes will be forced through a column (stationary phase) by introduction into the mobile phase. The neuro-

chemicals can be differentiated by different times at which they will interact with the stationary phase as they travel through the column. DA will be oxidized at a carbon surface within the electrochemical detector; the resultant current flow will be quantified and will be proportional to the amount of DA in the samples. After the experiment is completed, brains will be analyzed histologically to confirm placement in the prefrontal cortex. The data (pg DA/20 µl) will be expressed as a percentage of the last 3 baseline samples. It is predicted that the levels of DA observed in the prefrontal cortex will be higher in the wild-type mice than in the *Pet*-1 KO mice. This result would indicate that physiologically released 5-HT normally acts to stimulate the release of DA. As a result, the regulation of DA release by the actions of the 5-HT system will be more thoroughly understood, and pharmacotherapy will be advanced in the treatment of mood and cognitive disorders.

INTRODUCTION

Two major neurochemicals that regulate the behavior and emotions of animals are dopamine (DA) and serotonin (5-HT). An insufficient amount of serotonin in the brain has been associated with many mood disorders, including depression, and a variety of anxiety disorders (Nestler et al., 2001). Abnormalities in dopaminergic transmission have been implicated in illnesses such as schizophrenia and Parkinson's disease (Alex & Pehek, 2006). Furthermore, interactions between DA and 5-HT may play a role in the etiology/treatment of these disorders.

There are three main pathways that ultimately serve to release DA in the brain (Wolf et al., 1987). The mesolimbic pathway begins in the ventral tegmental area (VTA) of the midbrain and extends to the limbic system, including the nucleus accumbens. These neurons play a major role in memory, emotional responses, and rewards. The nigrostriatal pathway projects from the substantia nigra to the striatum, and regulates voluntary motor activity. The mesocortical pathway begins in the VTA and terminates in the prefrontal cortex. DA released at the terminus of this pathway regulates cognitive processes such as attention and memory (Alex & Pehek, 2006).

Serotonergic neurons arise in the raphe nuclei of the brain. These neurons project to many forebrain sites including the limbic system, striatum, and prefrontal cortex (Alex & Pehek, 2006). The cell bodies and terminal regions of the DA pathways are innervated by 5-HT neurons; studies have proven the existence of direct synapses between 5-HT terminals and DA cells in the midbrain (Herve et al., 1987; Nedergaard et al., 1988), suggesting a possible effect of 5-HT on DA neurons and DA production (Alex & Pehek, 2006).

One particular class of 5-HT receptors, known as 5-HT2A receptors, has been implicated in the regulation of the action of corticotegmental projection neurons which regulate the activity of DA neurons (Pehek et al., 2006). Thus, 5-HT2A receptors are believed to stimulate DA release in all three of the aforementioned DA pathways. In particular, DA release in the mesocortical system was shown to be increased upon stimulation of the cortical 5-HT2A receptors. These receptors have also been suggested to play a modulating role in DA release triggered by causes such as physiological stress (Devaud et al., 1992; Pehek, 1996; Pehek & Bi, 1997; Shi et al., 1995; Gobert & Millan, 1999; Pehek et al., 2001; De Deurwaerdere & Spampinato, 1999; Schmidt

& Fadayel, 1996; Zhang et al., 2000). 5-HT2C receptors have also been suggested to have an effect on DA activity; their localization in the brain (on DA receptors in the VTA) would suggest this role (Bubar et al., 2005; Ji et al., 2006). Evidence indicates that 5-HT2C receptors inhibit DA release in the striatum, nucleus accumbens, and prefrontal cortex of the brain (Alex & Pehek, 2006). The presence of dopamine and serotonin in the prefrontal cortex is particularly significant, because structures in this area of the brain have a profound influence on control of emotions, memory, and the response to rewards. Alterations in the levels of DA and 5-HT may be the cause of mood disorders such as schizophrenia, depression, and panic and anxiety disorders (Nestler et al., 2001).

The *Pet*-1 gene is a gene that has been found to be required for generation of normal serotonin neurons in the brain; the mice that lack this gene lack 80% of the normal amount of 5-HT neurons, and the neurons that are present in the adult mouse are generally defective. The *Pet*-1 gene is imperative for normal levels of anxiety and aggression. *Pet*-1 KO mice are deficient in neuronal tissue content of 5-HT as a result of the lack of 5-HT neurons in the forebrain. In mice that do not express the *Pet*-1 gene, most of the serotonin neurons do not differentiate, and the ones that do differentiate do not express the gene adequately for normal 5-HT production. Thus their behavior is significantly more impulsive, reflecting the lack of control over anger and anxiety due to the lack of 5-HT neurons (Hendricks et al., 2003).

While the tissue content of 5-HT is lower in *Pet* -1 KO mice, no studies have measured the actual extracellular release of 5-HT. Additionally, the lack of forebrain 5-HT in these mice may alter the amount of DA release. The present study examined the effects of neuronal 5-HT depletion (characteristic of *Pet*-1 KO mice) on extracellular release of DA and 5-HT by microdialysis in Pet-1 KO and wild type (WT) mice. Since the release of 5-HT in the brain has previously been shown to stimulate the release of DA, we hypothesized that the DA levels observed will be higher in the wild-type mice than in the KO mice, due to the higher amount of 5-HT present in the WT mice.

METHODS

Animals. The mice were PET heterozygote (+/-), KO (-/-), and WT (+/+) littermates generated by and obtained from Dr. Evan Deneris, Dept. of Neurosciences, Case Western Reserve University. Male and female mice were housed separately, with four mice per cage. There was a 12/12 hour light/dark cycle, and food and water were available ad libitum. All animal procedures were in strict accordance with the NIH Guide for the Care and Use of Laboratory Animals.

Surgery. Animals were first anesthetized via isoflurane (0.1 to 0.5% balance air to O_2). This anesthesia was employed throughout each surgery, and the level of anesthesia would be adjusted as needed. Using a stereotaxic instrument, a microdialysis probe was implanted into the prefrontal cortex of the mouse. Bregma was first located, and then the probe was implanted $\pm$0.300 mm laterally, 2.300 mm anteriorly and 3.500 mm ventrally (Paxinos & Franklin, 2004).

Microdialysis. *In vivo* microdialysis was employed using the implanted probe, in order to analyze the extracellular fluids (particularly the amounts of DA, 5-HT, and relevant metabolites) in the brain of the

mouse. The probes were of a concentric flow design with a 2.0 mm active dialyzing surface membrane (Carnegie-Medicin (CMA-7) MW cut-off = 6,000 Daltons, outer diameter=0.24 mm). The probe was connected to both inflow and outflow tubing. Through the inflow tubing, it was perfused with artificial cerebrospinal fluid (aCSF) that was devoid of the compounds that would ultimately be analyzed. In microdialysis, the compounds of interest diffuse from the extracellular fluid of the brain, through the dialysis membrane, into the aCSF pumped through the probe. Through the outflow tubing, the solution being perfused, along with the collected compounds from the brain, travel so that they can be collected (Shippenberg & Thompson, 1997). aCSF was prepared by adding 1.2 mM $CaCl_2$ and 5.0 mM glucose to Dulbecco's solution and was pumped through the probe using a Harvard infusion pump at a rate of 1.5 µL/min. Dialysate samples were collected from the mouse every 20 minutes. Baseline samples were recorded until the values (as shown by high performance liquid chromatography) of the neurochemicals/metabolites in the samples were stable over the course of 3 samples.

High Performance Liquid Chromatography (HPLC) with electrochemical detection. The physiological fluids collected from the mouse were analyzed using reverse phase HPLC. In HPLC, the compounds injected into the apparatus travel through a column which contains both a stationary and a mobile phase. HPLC is dependent upon the compounds being distributed differently between the stationary and mobile phases within the column of the machine. The compound injected into the apparatus is essentially dissolved in the mobile phase, and it moves through the column with the mobile phase (composed of 32 mM anhydrous citric acid, 54 mM sodium acetate trihydrate, 0.074 mM EDTA, 50 mg/L octylsulfonic acid, and 3% methanol; pH=4.2). However, different compounds interact to different extents with the stationary phase of the column, which is responsible for the differing elution times (times taken for different compounds to reach the detector). The compounds which interact the least with the stationary phase will exhibit the shortest elution times (Holman, 1993). The HPLC system consisted of a Schimadzu LC-ADvp solvent delivery system and a Bioanalytical Systems (BAS) LC-4C electrochemical detector equipped with a glassy carbon electrode. This electrode was maintained at a potential of +0.6 V relative to a reference electrode. Compounds were oxidized at the surface of this electrode and the current (electron flow) generated was converted to peaks. The respective height of each peak was proportional to the amount of the compound that it represented.

In this study, after standards containing DA and 5-HT as well as the metabolites 5-HIAA, HVA, and DOPAC were injected, the equipment was calibrated for the experiment based on the elution times and peak heights of the components of those standards. The samples of physiological fluids from the mouse (which contain the same compounds present in the standards) were then be injected onto a 2 x 100 mm Phenomenex (Torrance, CA) column (Ultracarb^{TM}, 3µm particle size, ODS 20). 20 µl of each sample were injected into the column. In cases of less than 20 µl of the sample being available, the amount present was injected, and an extrapolation was performed to estimate the amount of each neurochemical/metabolite in a normal 20 µl sample.

Histology. After the experiments are run, the mouse was euthanized via an injection of 0.3 mL of sodium pentobarbital. After this, the brain was removed, and the brain was sectioned with the aid of cryostat to ensure that the probe was placed properly during surgery. Slides were then stained with cresyl violet dye in order to clearly visualize the probe track. Data was discarded from mice with incorrect probe placements.

Experimental design. This study was divided into two experiments using the PET mice.

Experiment 1: Heterozygotes were used to determine whether DA, 5-HT, and metabolites could be successfully measured via *in vivo* microdialysis in the prefrontal cortex (PFC) of mice.

Experiment 2: The wild-type and knockout mice were compared following two different were compared following two different experimental treatments. In the control condition, the baseline data was collected, and then subsequent samples were collected in the same manner, without changing the environment of the mouse in any way. In the other treatment, the mouse was subjected to a mild stressor during the course of the experiment. During the second sample collected for the data, the mouse was stroked for the entire 20 minutes, as well as exposed to light (the cover normally placed on the cage was removed during this time).

RESULTS

Experiment 1. 4 PET heterozygotes (+/-) were used. DA, 5-HT, and the associated metabolites (DOPAC, HVA, and 5-HIAA) were shown to be measurable by *in vivo* microdialysis in the extracellular fluid of the PFC. Levels were relatively stable across time except for an unexpected increase in DA at time 20 (see Figure 1).

Experiment 2. Levels of DA, 5-HT, and metabolites were compared between WT and KO mice. The KO mice (n=3; average basal level 0.52 $\pm$ 0.16 pg/20 µl) did not appear to have a lower amount of DA than the WT mice (n=3; average basal level 0.47 $\pm$ 0.15 pg/20 µl). The levels of DA in each group over time are shown in Figure 2. The KO mice (n=3; average basal level 0.55 $\pm$ 0.08 pg/20 µl) also do not appear to have a

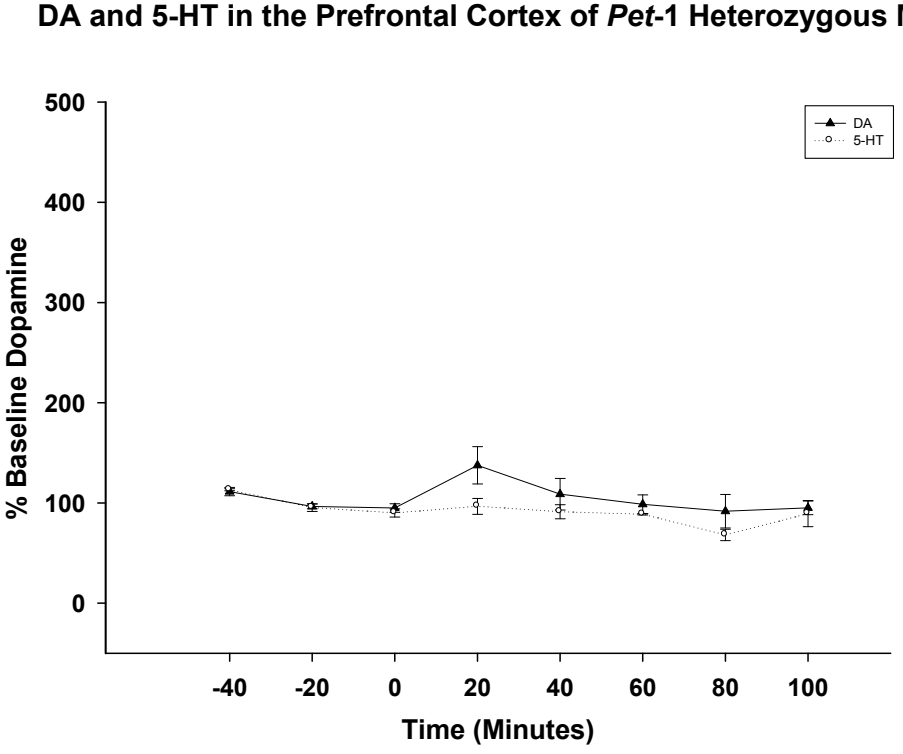

Figure 1. DA and 5-HT levels in PET +/- mice as taken from HPLC with electrochemical detection. The levels of DA and 5-HT are consistent with one another.

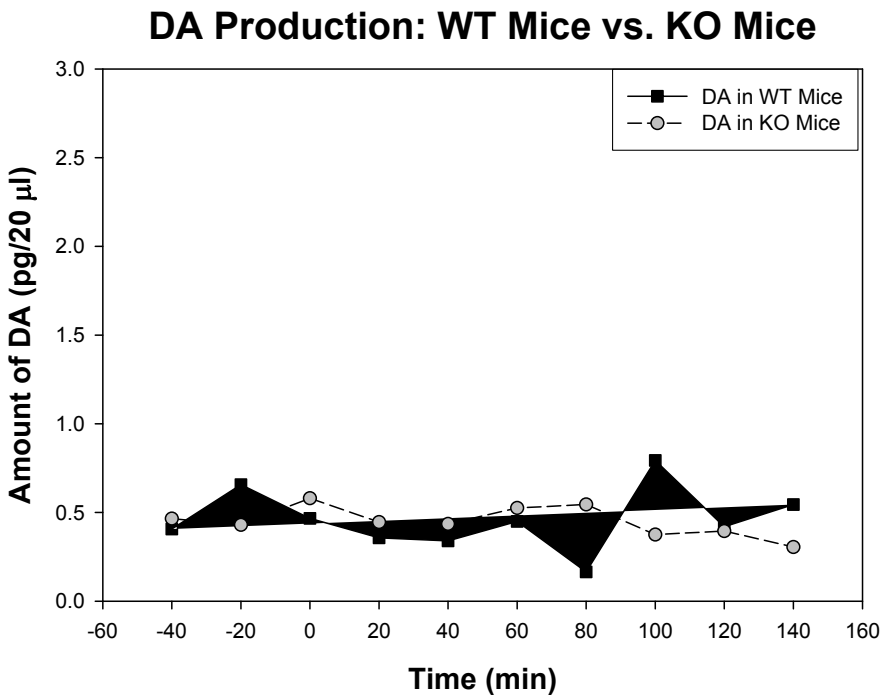

Figure 2. DA levels in WT and KO mice. The KO mice (preliminary data; n=3) do not appear to produce lower levels of DA than do the WT mice (preliminary data; n=3).

lower amount of 5-HT than the WT mice (n=3; average basal level 0.37 ± 0.12 pg/20 µl). The levels of 5-HT in each group over time are shown in Figure 3. The stress applied during the experiment did not appear to have an effect.

Though the differences between the groups in terms of DA and 5-HT appear to be insignificant at this point, there are differences in metabolite levels. The most significant difference is the amount of extracellular 5-HIAA; the KO mice (n=3; average basal level 11.03 ± 2.42 pg/5 µl) appear to produce significantly less 5-HIAA than do the WT mice (n=2; average basal level 66.80 ± 27.81 pg/5 µl) (see Figure 4). KO mice (n=3; average basal level 32.46 ± 5.56 pg/5 µl) appear to produce somewhat less DOPAC than do WT mice (n=2; average basal level 50.99 ± 17.93 pg/5 µl). Also, the KO mice (n=3; average basal level 142.54 ± 26.55 pg/5 µl) appear to produce significantly more HVA than do the WT mice (n=3; average basal level 90.58 ± 27.45 pg/5 µl).

DISCUSSION

The present results verified that *in vivo* microdialysis could be used to measure neurochemicals and metabolites in the mouse PFC. Interestingly, even though the levels of DA and 5-HT did not differ significantly between the two groups as expected, the levels of metabolites were different. The *Pet*-1 KO mice produced less extracellular 5-HIAA and DOPAC, and more HVA, than did the WT mice. The difference in 5-HIAA was the most noticeable and significant difference. Because of the small sample sizes thus far, more testing is needed to verify the significance of these trends.

Previous studies measuring neurochemicals in the brain have primarily used rats; microdialysis has been used to obtain results from the PFC of the rat (e.g. Pehek et al., 2006). Though extracellular neurochemicals had been quantified in other regions of the mouse brain, such as the nucleus accumbens core and striatum (Thomas et al., 2007), quantification in the PFC is difficult because of the relatively sparse innervation of this region of the brain with 5-HT and DA neurons.

While different studies have been done to elucidate the function of the *Pet*-1 gene (Hendricks et al., 2003), as well as the details of 5-HT neuron development (Gaspar, 2004; Stankovski et al., 2007), the role of

the *Pet*-1 gene in regulating neurotransmitter release has not been studied. While 5-HT tissue content is known to be depleted in *Pet*-1 KO mice (Hendricks et al., 2003), this is the first study to measure *extracellular* release. The results indicate that extracellular 5-HT levels are maintained despite a profound loss of 5-HT neurons in the knockout mouse.

While previous findings would suggest that there would be a lower amount of 5-HT measured in KO mice than in WT mice, the data from this study at this point do not support this assertion. The DA and 5-HT levels would be expected to be significantly lower in the KO mice than in the WT mice, but the amounts of these neurochemicals do not appear to be lower. However, the metabolites DOPAC, HVA, and 5-HIAA appear to be significantly different between the two groups. These results, though preliminary, indicate that the effect of the *Pet*-1 gene is exercised chiefly on the metabolites rather than on the extracellular levels of DA and 5-HT.

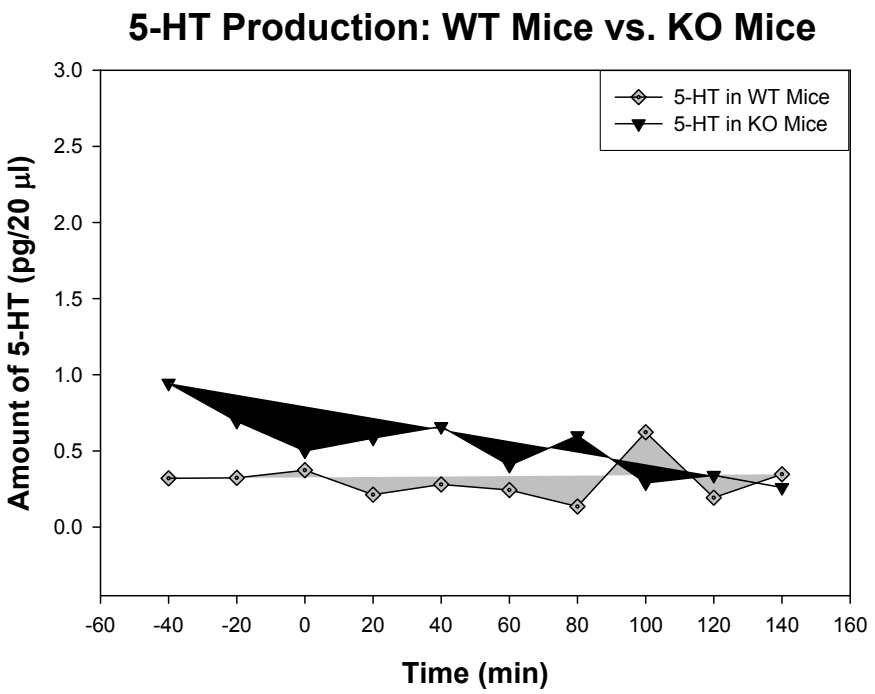

Figure 3. 5-HT levels in WT and KO mice. The KO mice (preliminary data; n=3) do not appear to produce lower levels of 5-HT than do the WT mice (preliminary data; n=3).

One major limitation to this study is the possibility that the fluids collected from the brain during microdialysis are not truly representative of the neurochemicals and metabolites being released by neurons, since there are other cells in the brain that release these chemicals. However, previous experiments in rats have shown that dialysate levels of DA and 5-HT are Ca^{2+}-dependent and increase in the presence of high K^+ (e.g. Moghaddam & Bunney, 1990), indicating that the measured release is truly neuronal. It is likely that dialysate DA and 5-HT are of neuronal origin in the mouse as well, although this should be tested in the future. Another limitation is that the deletion of the *Pet*-1 gene during development may have produced effects other than 5-HT neuronal loss that would confound interpretation of the results.

The difference in metabolites, particularly 5-HIAA, introduces a new possible direction to explore with these mice. It is likely that this effect is due to the depletion of 5-HT neurons in the *Pet*-1 KO genotype. The absence of the *Pet*-1 gene in the KO mice could cause a higher percentage of the 5-HT to be released from the neurons, which may explain the similar amount of 5-HT found in WT and KO mice. The gene may also alter the metabolism of 5-HT. Monoamine oxidase (MAO) is the enzyme that oxidizes 5-HT to

form 5-HIAA. It is possible that MAO may be inhibited in the knockout mice in order to preserve extracellular 5-HT concentrations. Reuptake inhibition is also a possibility. The preservation of extracellular 5-HT may be a mechanism present in order to keep the 5-HT levels as close to normal as possible, due to the inherent drive for homeostasis within the animal. Though the mechanism by which the number of 5-HT neurons would affect the amount of extracellular 5-HIAA is not currently known, it introduces the possibility of new implications that can elucidate factors that affect the release, metabolism, and reuptake of 5-HT and thus aid in providing new genetic and pharmacological treatments for mood disorders that are affected by the amounts of DA and 5-HT in the brain.

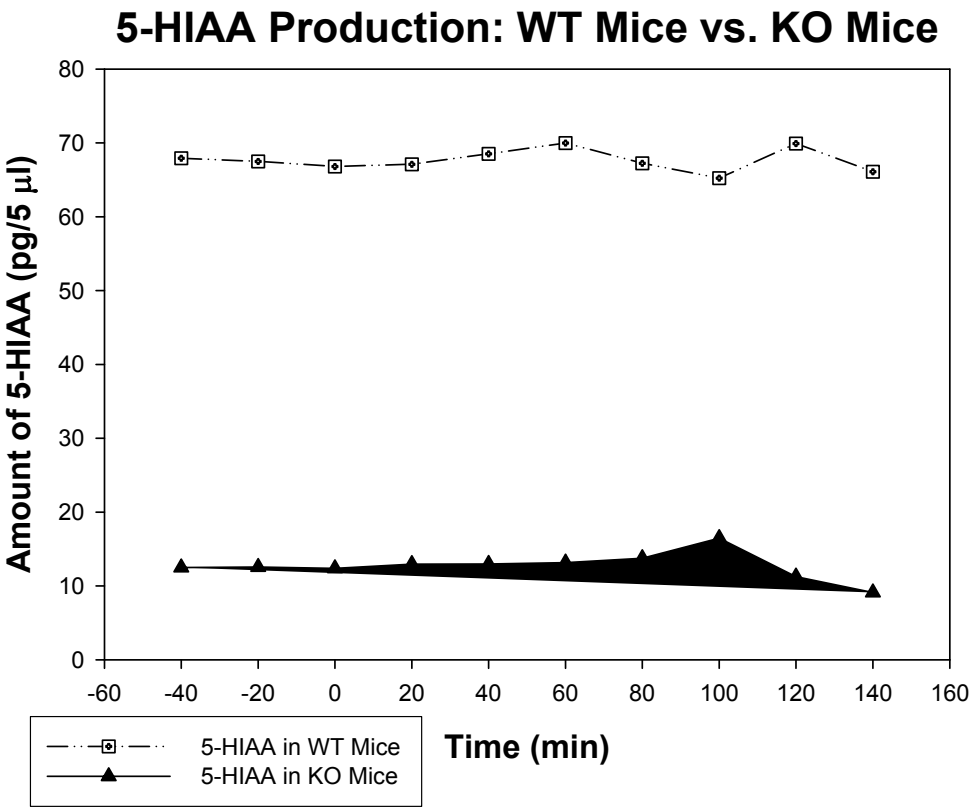

Figure 4. 5-HIAA levels in WT and KO mice. The KO mice (preliminary data; n=3) appear to produce significantly lower levels of 5-HIAA than do the WT mice (preliminary data; n=2).

REFERENCES

Alex, K.D. and E.A. Pehek. "Pharmacologic mechanisms of serotonergic regulation of dopamine neurotransmission." Pharmacology & Therapeutics 113 (2006), 296-320.

Bubar, M.J., et al. "Validation of selective serotonin 5-HT2C receptor antibody for utilization in fluorescence immunohistochemisty studies." Brain Research 1063 (2005), 105-113.

De Deurwaerdere, P. and U. Spampinato. "Role of serotonin2A and serotonin2B/2C receptor subtypes in the control of accumbal and striatal dopamine release elicited in vivo by dorsal raphe nucleus electrical stimulation." Journal of Neurochemistry 73 (1999), 1033-1042.

Devaud, L.L., E.B. Hollingsworth, and B.R. Cooper. "Alterations in extracellular and tissue levels of biogenic amines in rat brain induced by the serotonin$_2$ receptor antagonist, ritanserin." Journal of Neurochemistry 59 (1992), 1459-1466.

Gaspar, P. "Genetic models to understand how serotonin acts during development." Journal de la Société de Biologie 198 (2004), 18-21.

Gobert A. and M.J. Millan. "Serotonin (5-HT)2A receptor activation enhances dialysate levels of dopamine and noradrenaline, but not 5-HT, in the rat frontal cortex of freely-moving rats." Neuropharmacology 38 (1999), 315 -317.

Hendricks, T.J., et al. "Pet-1 ETS gene plays a critical role in 5-HT neuron development and is required for normal anxiety-like and aggressive behavior." Neuron 37 (2003), 233-247.

Herve, D., et al. "Serotonin axon terminals in the ventral tegmental area of the rat: fine structure and synaptic input to dopaminergic neurons." Brain Research 435 (1987), 71-83.

Holman, R.B. "Theoretical and Practical Aspects of High Performance Liquid Chromatography." High Performance Liquid Chromatography in Neuroscience Research (1993), 4-33.

Ji, S.P., et al. "Disruption of PTEN coupling with 5-HT2C receptors suppresses behavioral responses induced by drugs of abuse." Nature Medicine 12 (2006), 324-329.

Moghaddam, B. and B.S. Bunney. "Acute effects of typical and atypical antipsychotic drugs on the release of dopamine from prefrontal cortex, nucleus accumbens, and striatum of the rat: an *In Vivo* Microdialysis Study." Journal of Neurochemistry 54 (1990), 1755-1760.

Nedergaard, S., J.P. Bolam, and S.A. Greenfield. "Facilitation of a dendritic calcium conductance by 5-hydroxytryptamine in the substantia nigra." Nature (London) 333 (1988), 174-177.

Nestler E.J., S.E. Hyman, and R.C. Malenka. Molecular Pharmacology: A Foundation for Clinical Neuroscience (2001), 167-200.

Paxinos, G. and K.B.J. Franklin. *The Mouse Brain in Stereotaxic Coordinates*. Academic Press: San Diego, 2004.

Pehek, E.A. "Local infusion of the serotonin antagonists ritanserin or ICS 205,930 increases in vivo dopamine release in the rat medial prefrontal cortex." Synapse 24 (1996), 12-18.

Pehek E.A. and Y. Bi. "Ritanserin administration potentiates amphetamine-stimulated dopamine release in the rat prefrontal cortex." Progress in Neuro-Psychopharmacology & Biological Psychiatry 21 (1997), 671-682.

Pehek E.A., et al. "M100,907, a selective 5-HT2A antagonist, attenuates dopamine release in the rat medial prefrontal cortex." Brain Research 888 (2001), 51-59.

Pehek E.A., et al. "Evidence for the preferential involvement of 5-HT2A serotonin receptors in stress- and drug-induced dopamine release in the rat medial prefrontal cortex." Neuropsychopharmacology 31 (2006), 265-277.

Schmidt, C.J. and G.M. Fadayel. "Regional effects of MK-801 on dopamine release: effects of competitive NMDA or 5-HT2A receptor blockade." The Journal of Pharmacology and Experimental Therapeutics 277 (1996), 1541-1549.

Shi, W.X., P. Nathaniel, and B.S. Bunney. "Ritanserin, a 5-HT2A/2C antagonist, reverses direct dopamine agonist-induced inhibition of midbrain dopamine neurons." The Journal of Pharmacology and Experimental Therapeutics 274 (1995): 735-740.

Shippenberg, T.S. and A.C. Thompson. "Overview of microdialysis." Current Protocols in Neuroscience 7 (1997), 1-22.

Stankovski, L. et al. "Developmental cell death is enhanced in the cerebral cortex of mice lacking the brain vesicular monoamine transporter." The Journal of Neuroscience 27 (2007), 1315-1324.

Thomas T.C., et al. "Dopamine D4 receptor knockout mice exhibit neurochemical changes consistent with decreased dopamine release." J Neurosci Methods (2007).

Wolf, M.E., A.Y. Deutch, and R.H. Roth. "Pharmacology of central dopaminergic neurons." Handbook of Schizophrenia Neurochemistry and Neuropharmacology of Schizophrenia Vol. 2. Ed. F.A Henn and L.E. DeLisi. New York: Elsevier Science Publishers B.V., 1987.

Zhang, W., et al. "Synergistic effects of olanzapine and other antipsychotic agents in combination with fluoxetine on norepinephrine and dopamine release in rat prefrontal cortex." Neuropsychopharmacology 23 (2000), 250-262.

Impact of a Peer-Educator Led LifeSkills Training Tobacco Module on Tobacco-Related Attitudes, Knowledge, and Susceptibility in 5th and 6th Graders

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ABSTRACT

The purpose of this intervention was to assess student changes on measures of susceptibility, knowledge, and attitudes relating to smoking before and after administration of the peer-educator led Life Skills Training Program (LST) in the Shaker Heights Woodbury Upper Elementary School. Tobacco is the leading cause of preventable death in the United States, and it is estimated that 80 percent of tobacco users initiate use before the age of eighteen. Many public health intervention programs have been tailored to youth prevention efforts; the Life Skills Training Program (LST) is one such program. LST is a school-based intervention targeted at middle school aged children that can be taught by either teachers or peer-educators. A study population comprised of fifth and sixth graders from Woodbury School in Shaker Heights who would be receiving the tobacco module of the LST intervention was sampled. Students received a pre and post test survey. A total of 653 students participated. Age, gender and grade level were the only demographic data obtained. Results were stratified according to grade, gender, smoker in the household and previous tobacco use. Significant changes in attitudes and knowledge were seen between pre and post tests in all categories except girls' attitudes. Significant changes in susceptibility scores were only seen in half the categories. 5th graders showed lower pre test knowledge scores than 6th graders, but 5th graders also showed greater improvement in knowledge about tobacco between the pre and post tests compared to 6th graders. Girls and boys had comparable changes in knowledge. However, there was no significant change in girls' positive attitudes towards smoking. Students with prior smoking history showed less change in knowledge

and positive attitudes towards smoking. These groups also did not show a significant reduction in susceptibility between pre and post tests. Boys and students with a smoker in the house had greater susceptibility scores than other subgroups. The LST tobacco module appears to be effective in increasing knowledge and decreasing students' positive attitudes towards smoking. Susceptibility appears to be less affected by this intervention than attitudes and knowledge. However, based on our results, it may be beneficial for instructors or program designers to focus on certain subgroups who do not show significant changes in knowledge or susceptibility. Future research should be conducted to compare peer-led and teacher-led interventions, as well as to better understand whether differences exist in results based on gender of the peer instructors. It would also be beneficial to do a long term follow-up to further determine the impact of the intervention on later smoking behavior.

BACKGROUND

Tobacco is the leading cause of preventable death in the United States (CDC, 2002). Many public health initiatives and policies have focused on reducing overall tobacco use as well as prevention of smoking initiation. Approximately 80 percent of tobacco users initiate use by age eighteen, making adolescents the most vulnerable population (YTS 2000). In addition, a trend of increasingly early tobacco initiation has been observed, with 8.4% of middle school smokers trying smoking before they reach the age of 11 (YTS 2000). According to the Youth Tobacco Survey, 36.3% of middle school students have used tobacco at least once, while 15.1% currently smoke (YTS 2000). If the trend of early tobacco initiation continues, about five million U.S. children who are currently under 18 years old will die prematurely as adults due to tobacco-related causes (CDC 1996).

Tobacco Reduction education programs that seek to change social norms, to educate regarding the negative effects of smoking, and teach skills to resist smoking initiation are therefore recommended for young adolescents. LST is one such school-based program designed to prevent alcohol, tobacco, and other substance use among youth. The program has a three-pronged approach, aiming to address 1) alcohol, tobacco, and other substance use (ATOD)-related knowledge, attitudes, and norms; 2) skills for resisting social influences, and 3) personal self management skills. The LST program is comprised of 15 class periods, lasting approximately 45 minutes each, and aimed at middle school or junior high school students. The program also contains later booster sessions to reinforce the material throughout the students' academic experience. In all there are at least 10 booster sessions following the first year of the program, and five the following year.

LST is currently the most comprehensively evaluated school-based prevention program available. More than two decades of research have demonstrated prevention effects with respect to alcohol (Botvin et al, 1984,1994), tobacco (Botvin et al.1980, 1983), and marijuana use, other substance use, multiple substance use (Oetting, 1987; Botvin, 1990; Hawkins, 1992), and hypothesized mediating variables (Botvin 1982). The magnitude of the reported effects has usually been large, with most studies reporting reductions of 50% or more relative to control groups.

Current literature suggests that anti-smoking programs may be more effective if they employ peer

educators instead of classroom teachers. A recent literature review by Gottfredson & Wilson (2003) studied 94 other research reports and looked at peer-led interventions, teacher-led interventions, and integrated teacher-peer interventions in school-based substance abuse programs. The authors conclude that peer-only delivery had the highest effect size, while programs using peers and teachers, and teachers-only were significantly less effective. Therefore, the peer-teaching element in the LST program may be key in effectively conveying the anti-smoking message to students.

This paper therefore focuses on evaluation of one tobacco module of a peer-lead program in upper elementary students. Existing research studies have evaluated the LST program as a whole but have not determined the effectiveness of individual components. Our evaluation supplements existing research by providing data on the efficacy of one specific session of the LST program. This research will evaluate the effect of the tobacco module of the LST program on students' attitudes, knowledge, and susceptibility regarding tobacco.

METHODS

Design: This study utilized a quasi-experimental, one group pre test/post test design.

Participants: The study population was comprised of all fifth and sixth grade students at Woodbury Upper Elementary School in Shaker Heights, Ohio who received one peer-educator led tobacco-related LST module. Woodbury was selected for the program evaluation due to high sample availability and the racial and socioeconomic diversity of the student body. A total of 653 students were included, representing all students receiving this educational model from peer-educators.

Instruments: The students completed the pre test prior to the LST presentation and the post test after the presentation. The pre and post tests were matched according to pre assigned numbers, maintaining the anonymity of the subjects. Age, gender and grade level were the only demographic data obtained from the survey. The questionnaire used was a modified version of an existing LST evaluation instrument. Our version consisted of seventeen questions to assess knowledge, attitudes, susceptibility, and behavior. We also added a word bank to allow students a more open-ended way to express their attitudes regarding smoking.

Analysis: To compute knowledge scores, we used seven questions assessing the student's knowledge of a number of smoking related issues that were covered during the educational module, specifically: 1) whether or not they believed most adults smoke cigarettes, 2) if smoking causes your heart to beat slower, 3) if smoking makes it harder for kids to breathe, 4) if smoking makes one's teeth and fingers yellow, 5) if a stimulant is a chemical that calms down the body, 6) if smoking costs a lot of money, and 7) if they believed most kids their age have tried smoking. The data was recoded and dichotomized so that a student received a score of "1" for each correct answer and a score of "0" for each incorrect answer.

The responses were then summed to give each participant a knowledge score between 0 and 7 on both the pre and the post test. Paired sample t-tests were used to compare pre and post test results within each group, and independent sample t-tests were used to compare post test results between groups to determine magnitude of knowledge test score changes.

Questions that were included in attitude scores were: 1) do you think smoking cigarettes makes you look cool, 2) do you believe kids who smoke have more friends, and 3) do you believe that most kids your age have tried smoking? Like knowledge and susceptibility questions, these questions were combined to form an attitude score via recoding. A decrease in attitudes scores shows more negative attitudes regarding smoking. Although combining these three questions does not produce optimal internal consistency for either the pre test or the post test, the combined statistical power of these three questions combined was greater than evaluating each attitude question alone (Cronbach's alpha pre=.463, post=573). Each of the items included in this score contributes to the Cronbach's alpha, with any deleted item lowering the Cronbach's alpha, indicating that each item contributes to internal consistency.

In order to obtain the susceptibility scores, questions: 1) do you think you will smoke cigarettes or cigars within the next year, 2) do you think you will smoke cigarettes or cigars in high school, and 3) if one of your friends offered you a cigarette would you smoke it, were combined and recoded on a one to five point scale. The sums of the scores were divided by the total number of questions that the students answered to give each participant a susceptibility score between 1 and 5, with increasing numbers indicating increased susceptibility to smoking. We were able to combine these three questions with high reliability for both the pre and post test results (Cronbach's alpha= 0.776, 0.843).

To analyze the data, SPSS v.12.0 for Windows was used. We excluded all LST cases who did not complete the pre test or post test, totaling 29 cases. Our final analysis included 624 cases from the LST program (95.5% of total responses included in analysis). Results were stratified our by grade level, gender, current or past smoking behavior, and household smoker.

Hypotheses:

1. It was predicted that students would improve from pre test to post test in knowledge, attitude and susceptibility, with the greatest change occurring in knowledge, then attitude, then susceptibility.
2. It was predicted that personal exposure to tobacco use would make students less receptive to the teaching session, including 1) students with a smoker in the home, 2) students who reported current smoking versus those who did not and 3) students who reported smoking by at least one of their closest friends.
3. It was predicted that there would be differences in response based on gender and grade level.

RESULTS

Participant Demographics, shows the characteristics of the student population who participated in the LST program evaluation. The study population was ap-

	N	%	N	%
LST Total	624			
5th Grade/6th Grade	329	52.7	295	47.3
Boy/ Girl	326	52.2	272	43.6
Smoker/No smoker in home	166	26.6	453	72.6
Past/No past tobacco use	53	8.5	568	91.0

Table 1: Participant Demographics

	Pre test mean score	Post test mean score	t-test	p-value
Adults smoke cigarettes	0.56	0.81	-11.63	**<.001**
Smoking a cigarette causes your heart to beat slower	0.18	0.26	-3.74	**<.001**
Smoking makes it harder for kids to breathe	0.05	0.03	1.15	0.250
Smoking makes your teeth and fingers yellow	0.22	0.04	10.08	**<.001**
A stimulant is a chemical that calms down the body	0.52	0.56	-1.98	**0.049**
Smoking cigarettes makes you look cool	0.31	0.26	2.00	**0.047**
Kids who smoke have more friends	0.72	0.54	4.64	**<.001**
Smoking costs a lot of money	2.29	3.29	-15.06	**<.001**
Most kids my age have tried smoking	1.23	1.15	2.02	**0.044**
Do you think you will smoke cigarettes within the next year	3.83	3.87	-1.84	0.066
Do you think you will smoke cigarettes in high school	3.72	3.78	-2.44	**0.015**
If one of you friends offered you a cigarette would you smoke it	3.78	3.83	-2.70	**0.007**
Does anyone in your house smoke	0.26	0.25	1.00	0.318
Do any of your close friends smoke	0.07	0.08	-0.85	0.394
Have you ever tried smoking, even one or two puffs	0.05	0.04	0.45	0.655
Have you ever tried smoking cigars, cigarillos, or little cigars	0.03	0.05	-2.14	**0.033**

Table 2: Results of Individual Items from the Paired Sample T-test, LST Intervention Pre Test vs. Post Test

	n	Pre test mean score	Post test mean score	t-test	p-value
All Particiapnts					
5th Grade	327	3.86	5.08	-15.24	**<.001**
6th Grade	293	4.13	4.87	-9.94	**<.001**
Boy	324	4.06	5.04	-12.50	**<.001**
Girl	272	3.94	4.96	-12.51	**<.001**
Smoker in home	165	3.64	4.83	-10.19	**<.001**
No smoker in home	450	4.12	5.06	-14.94	**<.001**
Past tobacco use	53	3.92	4.68	-3.43	**0.001**
No past tobacco use	564	4.00	5.02	-17.75	**<.001**

Table 3: Changes in Knowledge Based on Personal Characteristics

proximately evenly distributed between both genders, with 52.2% of the participants being boys and 43.6% of the participants as girls. Approximately half, 52.7%, of participants were in 5th grade and 47.3% were in 6th grade. Since only 26.6% of participants reported having a smoker in the home, a majority of children do not experience tobacco use on a daily basis in their homes. Only 8.5% of study participants reported previous tobacco use.

Paired sample T-test, LST Intervention pre test vs. post test, shows the changes between the mean responses to questions on the pre and post tests administered to the LST participants. Most of the predicted questions asked showed significant changes in response. For example, between pre and post test, there was a significant change in the number of students who thought that most adults have smoked cigarettes (t=-11.63, p=<.001). When asked if smoking makes you look cool, question six, the mean decreased by 0.05 points between pre and post tests (t=1.995, p= .047) suggesting that fewer students felt that smoking makes you look cool after the LST intervention. Also, when students were asked if they believed if kids who smoke have more friends the mean decreased from 0.72 to 0.54 between pre and post test (t= 2.02, p=.044), indicating that between pre and post tests, less participants felt that their peers who smoke have more friends. Items that would not be expected to change between pre and post testing, such as household smokers, close friends smoking, and whether the student has tried smoking cigarettes, did in fact not generally change. Interestingly, there was a significant change in reports of cigar use from pre to post test, even though this was not a planned teaching goal.

As Described in Table 3, participants show significant changes in knowledge between the pre and post tests. (Report all participant total first) Overall 5th graders' mean knowledge scores increased statistically between the pre and post test from 3.86 to 5.08, (t=-15.24, p<.001). 6th graders knowledge scores also significantly increased from pre test scores to post test scores from 4.13 to 4.87 (t= -9.94, p< .001). Although 6th graders had a significantly higher knowledge score in the pre test questionnaires (t=-2.77, p=.006), by the post test the 5th graders significantly surpassed them in increased smoking knowledge (t=2.29, p=.023).

Boys showed a statistically significant increase in knowledge scores from 3.99 on the pre test to 4.98 on the post test (t (323) =-12.50, p<.05). Girls also showed an increase in mean knowledge scores from 4.06 to 5.04 (t (271) = -12.51, p< .05). At the time of the pre test and the post test both boys' and girls' knowledge scores did not differ significantly from one another (t=1.322, p=187), and (t= .896, p= .370) respectively.

Students who did not have a smoker in the home had a 4.12 pre test score and a 5.06 post test knowledge score showing a statistically significant increase in knowledge score (t= -14.94, p<.001). Participants who had a smoker in the home also showed a statistically significant increase, from 3.64 points to 4.83 knowledge score points in the post test (t=-10.19, p<.001). Those who did not have a smoker in the home had a significantly higher pre test score than those who do have a smoker in the home, (t=4.31, p<.001) and this carried over in to the post test as well, where students without smokers in the home still had a significantly higher post test score than those who did (t=2.23, p=.026).

Participants who reported no past tobacco use had a pre test score of 4.00 and a post test score of 5.02,

a statistically significant increase in mean knowledge scores (p=.001). Participants who did report past tobacco use also showed a significant knowledge point increase in mean knowledge scores from 3.92 to 4.68 from the pre test to post test p<.001). Students who did not use tobacco previously had significantly higher post test scores than those who had smoked previously.

Table 4 summarizes the participants' susceptibility scores, or their overall susceptibility to smoking initiation. (Report all participants first). 5th graders showed a decrease in mean susceptibility scores, from 1.18 in the pre test and 1.14 in the post test, but this decrease was only approaching significance (*t*=1.85, p=.065). However, 6th graders showed a statistically significant decrease in post test susceptibility scores, from 1.28 points in the pre test and 1.22 points in the post test (t=2.62, p=.009). 5th graders had a significantly lower pre test susceptibility score of 1.19 than 6th graders who had a pre test score of 1.28 (*t*(522.69)=-2.30, p<.05). 5th graders also had significantly lower post test susceptibility scores (1.14) than 6th graders (1.22).

	n	Pre test mean score	Post test mean score	t-test	p-value
All particiapnts					
5th Grade	324	1.18	1.14	1.85	0.065
6th Grade	293	1.28	1.22	2.62	**0.009**
Boy	324	1.28	1.22	2.87	**0.004**
Girl	269	1.65	1.13	1.79	0.075
Smoker in home	165	1.43	1.29	3.31	**0.001**
No smoker in home	450	1.15	1.14	1.17	0.244
Past tobacco use	52	1.77	1.65	1.50	0.140
No past tobacco use	564	1.18	1.14	2.82	**0.005**

Table 4: Changes in Tobacco Use Susceptibility Based on Personal Characteristics

Boys showed a statistically significant decrease in susceptibility from 1.28 in the pre test to 1.22 in the post test (*t*=2.87, p=.004). Also, girls showed a decrease from 1.16 in the pre test to 1.13 in the post test, indicating that girls' susceptibility was approaching statistical significance, (*t*= 1.79, p=.075). Girls had a significantly lower pre test mean of 1.28 susceptibility score than boys who had 1.17 (*t*=2.83, p= .005) and girls post test scores of 1.22 remained significantly lower than that of boys who had a mean score of 1.13 (*t*= 2.28, p=.023). Students who reported having a smoker in the home showed a significant point decrease from 1.43 to 1.29 in susceptibility scores (*t*=3.31, p=.001). Those who did not have a smoker in the home did not show a significant change in susceptibility due to the LST program. Students who had smokers in their houses entered with significantly higher susceptibility scores of 1.43 in comparison to those who did not have smokers in the home (1.16). Additionally, in post test susceptibility scores, students with no smoker in the home had significantly lower scores (1.14) when compared those with smokers in the home who had post test scores of 1.29.

Students who reported past tobacco use showed a decrease in mean susceptibility scores from 1.77 susceptibility points to 1.66 points, although this change

was not statistically significant (t= 1.5, p=.140). Those who had not used tobacco previously showed a significant decrease in mean susceptibility scores from 1.18 points to 1.14 (t= 2.82, p=.005).

Table 5 shows changes in students' attitude scores. 5th graders showed a significant decrease in positive attitude towards smoking with a pre test mean of 1.69 and a post test mean of 1.60 on the attitude scale (t= 2.84, p= .005). Also, 6th graders showed a significant decrease in positive tobacco attitude from 1.82 to 1.70 (t= 3.98, p<.001). Although 5th and 6th graders' pre test scores were significantly different from one another, with 5th graders' scores being lower, (t=-2.49, p=.013), by the post test, 6th graders' attitudes were not significantly different from 5th graders (t=-1.82, p=.069).

Boys' positive tobacco attitude scores significantly decreased from pre test to post test, from 1.75 to 1.60 (t=4.35, p<.001). Girls' post test attitude scores also decreased from 1.75 to 1.69, but this finding was not significant (t=1.78, p=.078). Boys and girls started with identical pre test attitudes scores.

Participants who reported a smoker in the home showed a significant decrease in mean attitude scores from 1.95 to 1.81 (t=2.93, p=.004). Those who did not have a smoker in the home showed a decrease in mean attitudes scores from 1.68 to 1.58 (t=3.81, p=.004). Those who did not have a smoker in the home had significantly lower pre test attitude scores (t=-4.25, p<.001) as well as post test scores (t=-3.63, p<.001).

Participants who reported past tobacco use showed a decrease in positive attitudes toward smoking, from 2.2 to 2.02 (t=2.22, p=.031). Students who reported no prior tobacco use also showed a significant decrease in negative attitudes from 1.70 to 1.60 (t=4.25, p<.001). Those who had never smoked had significantly lower pre tests attitude scores than those students who had smoked (t=-4.39, p<.001). This was true of post test scores as well; those who had never smoked had significantly lower scores than those who had (t=-3.12, p<.001).

	n	Pre test mean score	Post test mean score	t-test	p-value
All participants					
5th Grade	325	1.69	1.60	2.84	**0.005**
6th Grade	291	1.82	1.70	3.98	**<.001**
Boy	321	1.75	1.60	4.35	**<.001**
Girl	271	1.74	1.69	1.77	0.078
Smoker in home	165	1.95	1.81	2.90	**0.004**
No smoker in home	446	1.68	1.58	3.81	**<.001**
Past tobacco use	53	2.23	2.02	2.22	**0.031**
No past tobacco use	560	1.70	1.60	4.25	**<.001**

Table 5: Changes in Attitudes about Smoking Based on Personal Characteristics

Pre test			Post test		
Word	n	%	Word	n	%
1. unhealthy	477	76.4	1. unhealthy	447	71.7
2. dangerous	419	67.1	2. dangerous	401	64.3
3. stupid	417	66.8	3. stupid	377	60.4
4. addictive	213	34.1	4. expensive	203	32.5
5. smelly	134	21.5	5. addictive	198	31.7
6. expensive	76	12.2	6. smelly	141	22.6

Table 6: LST Word Bank

Table 6 examines the Tobacco Word Bank and demonstrates the top five responses on the pre and post tests. On both the pre and post tests, the most popular choices were "stupid" (pre=66.8%, post=60.4%), "dangerous" (pre=67.1%, post=64.3%) and "unhealthy" (pre= 76.4%, post= 71.7%). Interestingly, 20.3% more students chose "expensive" on the post test than the pre test. To compensate for this increase, the words stupid, unhealthy, dangerous, and addictive decreased slightly.

Finally, when comparing the Pearson's correlation coefficients of pre test and post test scores, we noted that the mean susceptibility score is positively and significantly related to attitudes. $r(615)=.294$, $p<.05$). Post test susceptibility scores are negatively and significantly correlated to post test knowledge scores $r(617)= -.137$, $p<.05$)., $r(616)=.279$, $p<.05$) and a .299 point increase in pre test knowledge scores, $r(617)=-.299$, $p<.05$). Post test attitude scores are significantly correlated to post test susceptibility scores and post test knowledge scores, $r(615)=.294$, $p<.05$) and $r(619)=-.37$, $p<.05$) respectively.

Finally, when examining Pearson's correlation coefficients in Table 7, post test values for positive tobacco attitude and future tobacco use susceptibility are significantly correlated with each other, and negatively correlated with knowledge. This implies that increased knowledge may have a role in determining attitudes and susceptibility in a manner that may decrease future tobacco use.

DISCUSSION

Prior research shows that knowledge and attitudes are often influenced by presentations such as LST, but susceptibility—the better predictor of future smoking behavior—is less easily influenced (Pierce et al. 1996). Therefore, we predicted that when questions were grouped according to knowledge, attitudes, and susceptibility, knowledge and attitudes would change most significantly. This was indeed true. When results were stratified according to grade, gender, smoker in household and prior tobacco use, significant changes in knowledge and attitudes were seen across all categories, but significant changes were only seen across half of the susceptibility categories. The only exception to this trend is girls' attitudes, which did not change significantly.

A particular finding of interest is the difference in pre and post test knowledge scores among 5th and 6th graders. While 5th graders' pre test knowledge scores were lower than 6th graders' scores, the 5th graders actu-

ally had higher scores than the 6th graders on the post test. Although both groups had significant changes in knowledge, the 5th graders learned and retained approximately 40% more knowledge than 6th graders, surpassing their older peers' post test knowledge scores even with lower tobacco-related knowledge scores at baseline. Future research should focus, therefore, on the reasons for this disparity and on possible methods for increasing 6th graders' knowledge scores even further. Perhaps the knowledge segment of the program needs to be administered differently to the two grades. Additionally, research should be conducted to see what the change in knowledge and attitudes is between 6th graders who have received a tobacco program in both 5th and 6th grade and those that receive a tobacco program in only 6th grade.

Girls' knowledge change and boys' knowledge change was comparable. However, while there was no significant change in girls' positive attitudes toward smoking, boys' positive attitudes did change significantly. More research should be conducted to examine the reasons for these differences in attitude change. Perhaps an additional attitude-related component of LST should be geared specifically toward girls.

Students without prior tobacco use gained 25% more knowledge than those students with prior tobacco use. However, those students with prior tobacco use had decrease in positive attitudes towards smoking that was more than twice that of those of students without prior tobacco use. Additionally, our results on the susceptibility portion of the survey indicate that while most subgroups reported a significant reduction in susceptibility to smoking, current or past smokers did not. However, the insignificance of this groups' reduction may be attributable to the smaller size of the group (n=56), as their numeric scores actually changed more than those of non-smokers. We therefore recommend that more attention be paid to this particularly susceptible subgroup to assess how much they are truly benefiting from LST. The LST curriculum currently does not include any section geared toward current or past smokers; perhaps a brief discussion focusing on the benefits of quitting if a student already smokes would be helpful. Additionally, LST presenters could mention a few resources for students who wish to quit smoking. An additional study with a much larger sample size of current or past smokers would help clarify the program's true impact on this population.

Other particularly susceptible subgroups included boys and students with a smoker in the household. While these groups did have a significant decrease in susceptibility, their post test susceptibility scores were still much higher than the pre test and post test scores of less susceptible groups. Thus, while the LST program was effective in significantly reducing the susceptibility scores of these groups, future research should focus on reducing these groups' susceptibility scores even further.

Lastly, while the 5th graders did not have a significant decrease in susceptibility scores, this lack of change was primarily due to their low pre test score (1.14). In contrast, the 6th graders showed a statistically significant decrease in post test susceptibility scores, from 1.28 points in the pre test to 1.22 points in the post test. However, it is notable that their post test scores were still higher than 5th graders' scores on the pre test. This highlights 6th graders as a more susceptible group; perhaps more research should focus on affecting this older cohort in particular.

Limitations: Our study had several limitations. Most importantly, there was no short or long term follow-up, so we cannot draw any conclusions about lasting effects of the LST program on students' tobacco knowledge, attitudes, and susceptibility. Additionally, we had no way to determine the extent to which classroom teachers participated in LST presentation. This limitation is of particular concern; many studies have shown significant differences in the effectiveness of teacher-led versus peer-led anti-smoking interventions. Our study also did not include any questions about socioeconomic status or race/ethnicity. This is problematic since these factors may affect smoking susceptibility. Lastly, while many of our questions were from previously tested surveys, the word bank was not from literature, and was not framed in a manner that facilitated pre test/post test analysis. The other questions from prior literature did not all come from the same source, and therefore had not been used in combination before. Study strengths included a large study population and standardized questionnaire administration. Additionally, while we did not ask students to report race or ethnicity, all students came from one school with a very diverse student body.

Future Research: Future research should include long-term follow-up to determine if the LST intervention does in fact deter smoking behavior. It is also important to determine differences in results between classes that have only peer educators and those that have a teacher influence. Additionally, anecdotal evidence shows there to be a possible difference in effectiveness between male and female peer-presenters. Future research should examine this difference. Lastly, it is important to determine the extent to which peer administration of LST aided or hindered program effectiveness. Comparing a peer-led LST program with an adult-led program would help clarify this issue.

RESOURCES

Botvin GJ, Sussman S, Biglan A. "The Hutchinson Smoking Prevention Project: A Lesson on Inaccurate Media Coverage and the Importance of Prevention Advocacy (Editorial)." *Preventative Science,* 2001, 2:67-70.

Botvin GJ, Eng A. "The efficacy of a multicomponent approach to the prevention of cigarette smoking." *Preventive Medicine*, 1982, 11: 199-211.

Botvin GJ, Eng A., Williams CI. "Preventing the onset of cigarette smoking through Life Skills Training." *Preventive Medicine*, 1980. 9: 135-143.

Botvin GJ, Baker E, Botvin EM, Filazzola AD, Millman RB. "Alcohol abuse prevention through the development of personal and social competence: A pilot study." *Journal of Studies on Alcohol*, 1984a. 45: 550-552.

Botvin GJ, Schinke SP, Epstein JA, Diaz T. "The effectiveness of culturally-focused and generic skills training approaches to alcohol and drug abuse prevention among minority youth." *Psychology of Addictive Behaviors*, 1994. 8: 116-127.

Centers for Disease Control and Prevention (2002) MMWR "Annual Smoking–Attributable Mortality, Years of Potential Life Lost, and Productivity Losses — United States, 1995–1999". Vol. 51.

Centers for Disease Control and Prevention (1996) Morbidity & Mortality Weekly Report, November, 2006.

Gottfredson DC, Wilson DB. "Characteristics of Effective School-Based Substance Abuse Prevention." *Prevention Science*, 2003. 4:1, 27-38.

Hawkins JD, Catalino RF, Miller JY. "Risk and protective factors for alcohol and other drug problems in adolescence and early adulthood: Implications for substance abuse prevention." *Psychological Bulletin*, 1992. 112 (1), 64-105.

Oetting ER, Beauvais F. "Peer cluster theory, socialization characteristics, and adolescent drug use: A path analysis." *Journal of Consulting and Clinical Psychology*, 1987. 34:205-213.

Pierce JP, Choi WS, Gilpin EA, Farkas AJ, Merritt RK. Validation of susceptibility as a predictor of which adolescents take up smoking in the United States. *Health Psychology* 1996; 15(5): 355-361.

Mahoney MC, Bauer JE, Tumiel L, McMullen S, Schieder J, Pikuzinski D. "Longitudinal impact of a youth tobacco education program." *BMC Family Practice, 2002, 3:3.*

"Youth Tobacco Surveillance – United States, 2000." MMWR: Surveillance Summaries. November 02, 2001 / 50 (SS04);1-84.

Effects of *M. tuberculosis* antigens on CD4+ T cells

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ABSTRACT

One-third of the world's population is infected with *M. tuberculosis* (TB), of which the vast majority is latently infected (1). A 5% lifetime risk exists of latent infection becoming active disease (2). A positive purified protein derivative (PPD) skin test suggests previous or latent TB infection. We hypothesize that PPD positive individuals have a higher frequency of TB specific lymphocytes than PPD negative individuals, who are presumed to be uninfected. CD4+ T cells are the most important lymphocyte type in controlling TB infection. For these studies, CD4+ T cells were stimulated by monocytes, which are antigen presenting cells (APCs). APCs digest, process, and present antigens to CD4+ T cells in a form that CD4+ T cell receptors can recognize. CD4+ T-cells and monocytes were isolated by immunomagnetic bead methods. These purified cell populations were stimulated with different TB antigens including live TB, PPD, and whole cell TB lysate. Pro-inflammatory cytokine production (TNF-alpha, IL-2, and IFN-gamma) and CD154 expression were measured in each individual's cells by flow cytometry to determine the frequency of responding cells. TB activated cells express CD154, a protein that co-stimulates CD4+ T cells and APCs. As hypothesized, PPD positive individuals had a higher frequency of responding cells than PPD negative individuals. This more frequent response from PPD positive individuals suggests that these cytokine producing CD154-expressing CD4+ T cells were indeed TB antigen specific. PPD negative individuals have less TB specific CD4+ T cells since these individuals were presumably never infected with TB and thus their immune systems never developed a memory response to the bacilli.

INTRODUCTION

M. tuberculosis (TB) is a bacillus that causes the infectious disease tuberculosis. Tuberculosis infects over one-third of the world population. The majority of these people have a latent infection, meaning it is not active. About a 5% chance exists for the latent form to turn into active disease. TB is an aerobic bacterium that mostly affects the pulmonary tract. It divides every 16 to 20 hours, much slower than most other bacteria. TB is known as an opportunistic infection (OI) (3). OI are infections that generally do not cause the disease in people with healthy immune systems but in those with a poorly functioning or suppressed immune systems. HIV increases the risk of developing OI because the virus attacks the immune system and compromises its ability to respond to infections. TB is an OI that infects HIV+ individuals earlier in the course of HIV disease than any other OI, even before the HIV is detected in the individual. TB also infects individuals with a healthy immune system making it a more virulent OI than most of the others in this group. TB activated cells express CD154, a protein that co-stimulates CD4+ T cells and antigen presenting cells (APC). In studying the expression of CD154 in PPD positive versus PPD negative individuals, we hypothesize that PPD positive individuals have a higher frequency of TB specific lymphocytes than PPD negative individuals, who are presumed to be uninfected.

METHODS AND MATERIALS

PBMC Isolation:

The human subjects protocol was approved by the Institutional Review Committee at University Hospitals and Case Western Reserve University and informed consent was obtained from all donors. Blood was drawn from PPD positive and PPD negative individuals. Peripheral Blood Mononuclear Cells (PBMC) were purified by Ficoll (Pharmacia) per the manufacturer's instructions.

APC and T cell Purification:

CD14+ monocyte isolation was performed using immunomagnetic bead methods (Miltenyi) according to the manufacturer's instructions. CD4+ T cells were isolated using immunomagnetic Dynal bead methods as per the manufacturer's instructions (Invitrogen).

T cell Assays:

Purified CD4+ T cells (5x105/well) were incubated with CD14+ monocytes (5x105/well) in 96 well plates. All assays were performed in standard medium which consisted of RPMI 1640, supplemented with 2% pooled human serum (Gemini Bio-Products), 100 U/ml penicillin, 100 Ag/ml streptomycin, 1% non-essential amino acids, 10 mM HEPES, 2-ME (5 _10_ 5 M), and 2 mM glutamine (Cambrex). Six wells were used per donor with the following antigens diluted in each well: Media, TB 5 x 106(ATCC), TB 1 x 106, PPD (Wyeth Pharmacuticals), TB Lysate (CO State TB Contract), and SEB (staphylococcal enterotoxin B, Sigma). Anti- CD 28/49 at 0.5 ug/ml (BDBiosciences) was added to each well for costimulation. Brefeldin A 10 ug/ml (Sigma) was added after overnight culture and then cells were incubated for four hours before harvest.

Intracellular Staining:

Cells were incubated with the surface antibodies, anti-CD4-FITC (BDBioscicnes) and anti-CD154 -APC (Ebiosciences), for 15 minutes at room temperature. After fixation in 4% paraformaldehyde, cells were incubated in 0.1% saponin (Sigma) with anti TNF-alpha

-PE, IL-2-PE, and IFN-gamma-PE (all 3 EBiosciences) for 20 minutes at room temperature for intracellular staining. Cells were finally re-suspended in 2% paraformaldehyde and analyzed using flow cytometry within 24 hours. Pro-inflammatory cytokine production (TNF-alpha, IL-2, and IFN-gamma) and CD154 expression were measured by flow cytometry to determine the frequency of responding cells.

CD154+ cell Purification:

In selected experiments, CD154+ cell isolation using the immunomagentic anti-CD154 bead kit (Miltenyi was performed as per the manufacturer's instructions. Cell purity was determined by anti-biotin-APC (Miltenyi) staining of CD154 selected cells

RESULTS

To confirm our basic hypothesis that that PPD positive individuals have a higher frequency of TB specific lymphocytes than PPD negative individuals we obtained blood from 4 PPD- and 6 PPD+ donors. Monocytes and CD4+ T cells were purified from their blood using immunomagentic bead methods. The cells were then incubated with TB antigens overnight and the levels of pro-inflammatory cytokines and surface CD154 expression were determined by flow cytometry. The cells could either express CD154 alone, cytokines alone or be dual expressing. Using flow cytometry we were able to determine this on a per cell basis. Figure 1 below shows flow cytometry data for a PPD+ and a PPD - individual. As can be seen there is a clear difference in CD154 and cytokine expression.

Results for all individuals are shown as percentage positive expression in the CD4+ cells for each of the groups. Table 1 summarizes these results for all individuals tested. Also included is the percentage of total CD4+ T cells that expressed CD154 whether there was cytokine production or not. PPD positive individuals had a significantly higher frequency of cells that responded to the mycobacterial antigens than the PPD negative individuals for many of the TB antigens.

This table shows that on average, PPD positive individuals had a higher frequency of CD154+ cells, cytokine-producing cells, and cells that were both CD154+ and cytokine producing. The two different con-

		PPD- Individuals Mean (SD)	PPD+ Individuals Mean (SD)	p value
No Antigen				
	CD154	0.20 (0.02)	0.15 (0.12)	0.30
	cytokine	0.63 (0.67)	0.81 (0.72)	0.70
	Total CD154	0.23 (0.03)	0.47 (0.82)	0.50
	Dual	0.03 (0.03	0.01 (0.01)	0.34
TB High Dose				
	CD154	3.40 (1.31)	7.48 (4.87)	0.10
	cytokine	0.47 (0.25)	1.65 (0.81)	0.01
	Total CD154	3.57 (1.35)	8.18 (5.10)	0.08
	Dual	0.16 (0.08)	0.71 (0.29)	0.00
TB Low Dose				
	CD154	3.42 (2.26)	8.49 (5.22)	0.07
	cytokine	0.88 (1.11)	3.45 (2.25)	0.04
	Total CD154	3.53 (2.22)	10.08 (5.99)	0.05
	Dual	0.10 (0.07)	1.59 (1.22)	0.03
PPD				
	CD154	0.23 (0.07)	1.79 (1.04)	0.01
	cytokine	1.73 (1.99)	2.56 (1.36)	0.50
	Total CD154	0.27 (0.03)	2.46 (1.41)	0.01
	Dual	0.04 (0.04)	0.68 (0.42)	0.01
TB Lysate				
	CD154	0.62 (0.93)	1.42 (0.76)	0.21
	cytokine	1.06 (1.21)	1.75 (0.50)	0.34
	Total CD154	0.65 (0.93)	1.69 (0.94)	0.13
	Dual	0.02 (0.02)	0.27 (0.21)	0.03
SEB				
	CD154	2.34 (3.23)	8.58 (3.11)	0.02
	cytokine	2.88 (1.80)	11.97 (4.13)	0.00
	Total CD154	2.77 (3.91)	17.35 (10.97)	0.02
	Dual	0.43 (0.68)	6.35 (4.54)	0.02

Table 1. Mean cell expression of PPD positive versus PPD negative individuals. Mean values for PPD positive and PPD negative individuals are displayed on the left side of the respective columns and standard deviation is given in parenthesis. The last column gives P-values.

centrations of live TB had the highest and most significant differences between the two groups. The P-values for the majority of the different cell types are under 0.05. This means that there is less than a 5% chance that the results were accidental, meaning that the results were not a matter of chance, they were obtained through the proper methods.

Staphylococcal enterotoxin B (SEB) was supposed to function as the control, since it was an antigen not related to TB. SEB should have yielded similar values for both PPD positive and PPD negative individuals. However, overall, the number of cells activated by SEB were higher for PPD positive individuals than for PPD negative individuals.

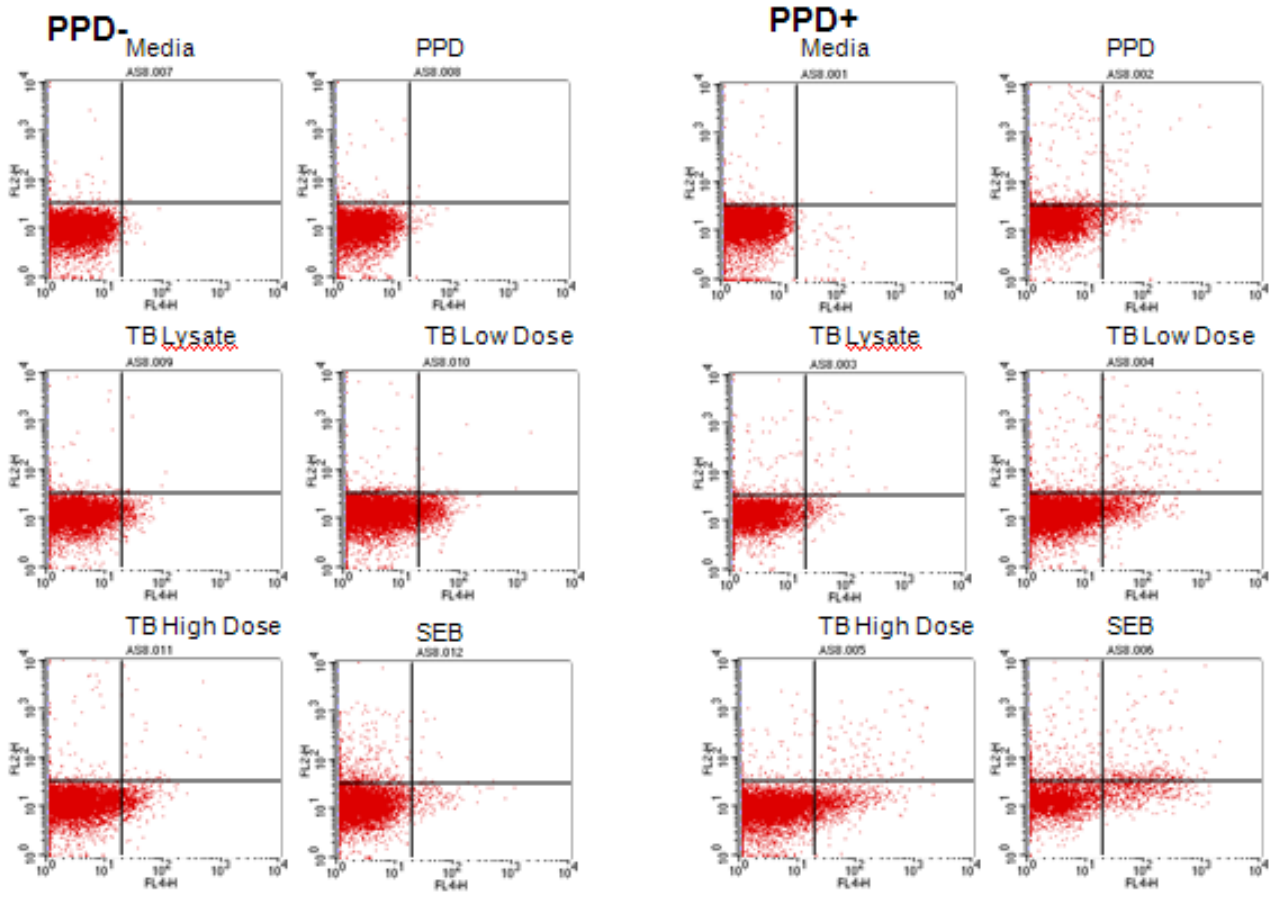

Figure 1: Sample flow cytometry results of a PPD- and a PPD+ donor. The six different environments cells were incubated in overnight were Media (no antigen), TB High dose, TB low dose, PPD, TB Lysat, and SEB. The PPD negative individuals produced a lower number of pure CD154+ cells (lower right quadrant), cytokine-producing cells (upper left and upper right quadrants), all CD154+ cells that did and did not produce cytokines (upper right and lower right quadrants), and CD154+ cells that produced cytokines (upper right quadrant). The PPD positive individual produced a larger number of cells for the aforementioned four cell types as can be determined from abundance of cells in the upper right, upper left, and lower right quadrants of the PPD negative and PPD positive individual results above. The x-axis represents CD154+ cell production. Cells in the lower left quadrant are not CD154+. The y-axis reprsents the production of the three cytokines, TNF-alpha-PE, IL-2-PE, and IFN-gamma-PE.

Figure 2 shows the actual data for every donor that was studied. It is apparent that the PPD- donors (Fig 2A) had a very low frequency of cells that were positive for CD154 or cytokines. PPD negative individuals also responded modestly to the antigens. Even when stimulated with no antigen, the frequency of production of the measured cell types was very low. This means that the PPD negative individuals produced very little CD154 overall.

The PPD positive donors' results (Fig 2B) show these individuals had an overall stronger response to the different antigens. SEB and the two doses of TB yielded in the highest cell responses. PPD and RVL were less effective at stimulating signal but as shown in Table 1 still had significant differences in most cases. These individuals had a higher percentage of CD154+, cytokine producing CD4+ T cells.

CD154+ cells were purified in selected PPD+ donors after stimulation with live TB by a two-step anti-CD154 immunomagentic bead kit. The yield could then be calculated by knowing the number of cells present before CD154+ purification after and the percent CD154+ cells as determined by the data in Fig. 1B. Yield was over 90% on the two donors shown. The purity of the CD154+ cells was determined by using anti-biotin-APC antibody. The purity was not that great as determined by this method. It is likely an underestimation of the purity of the cells as the biotin epitopes may have been obscured in the purified T cells by the anti-biotin beads used in the purification process.

DISCUSSION

As Table 1 and Fig. 2 show, PPD positive individuals expressed more CD154+, CD4+ T cells than PPD negative individuals did. They also produced more pro-inflammatory cytokines than PPD negative individuals did. The more frequent response from PPD positive individuals suggests that these cytokine producing CD154-expressing CD4+ T cells were indeed TB antigen specific. PPD negative individuals have less TB specific CD4+ T cells since these individuals were presumably never infected with TB and thus their immune systems never developed a memory response to the bacilli. This was the anticipated result. For this project, we wanted to develop the method to stimulate the TB specific cells and then try to purify the responding CD154 cells when they were live. This was the rationale in using the CD154 purification method described in Table 2. It has been demonstrated that CD154 up-regulation is a reasonable way to obtain live antigen specific cells (4-6).

With this knowledge, we hope to stimulate HIV+ cells with TB and observe the CD154+, CD4+ T cell frequency responses. We will then go on and purify the TB specific cells using this CD154 method that we have developed and confirmed. Since TB infects HIV+ individuals at a high rate, carrying out such experiments may yield an answer as to why this occurs. HIV+ individuals should be expected to have a lower frequency of TB specific CD 154+, CD4+ T cells since these individuals are unable to battle the infection effectively.

Further developing and characterizing these techniques will allow us to study responses in HIV infected individuals. We hypothesize that MTB specific CD4+ T cells are preferentially infected with HIV. This would

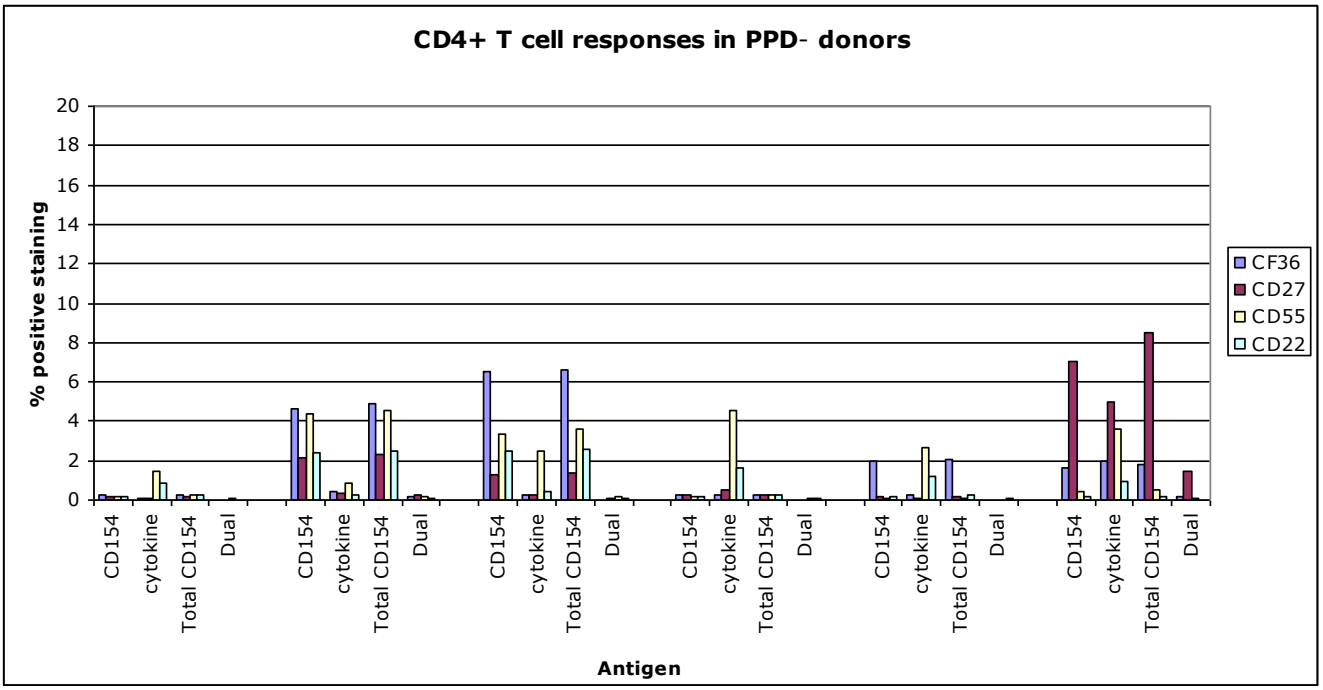

Figure 2A. CD4+ T cell responses to TB antigens in PPD negative donors. Each donor's cells were placed in six different antigens, with one having no antigens as a negative control, shown on the x-axis. The antigens used were TB at two different concentrations, PPD, TB Lysate, and SEB. The x-axis also shows the four different types of cell expression that were stained for and measured; pure CD154+ cells, cytokine-producing cells, all CD154+ cells that did and did not produce cytokines, and cells that were CD154+ and produced cytokines. The four cell types have been shown for the six different environments the cells were incubated in. The percentage of cells and types of cells that were activated by each of the individual antigens is shown.

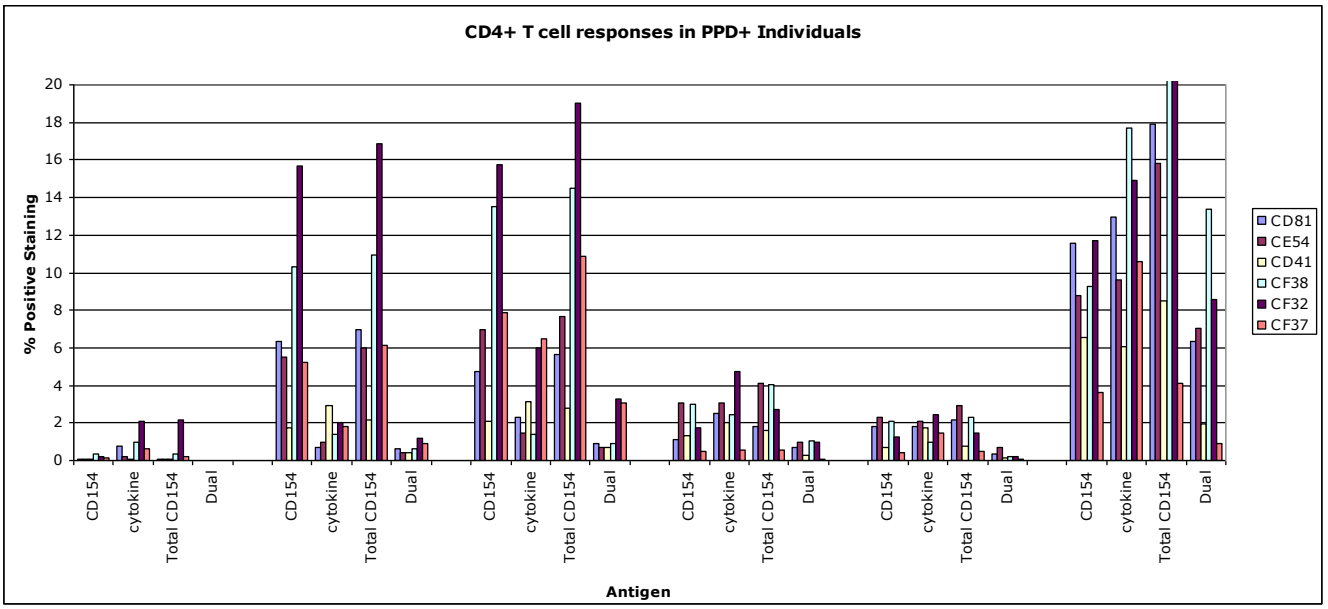

Figure 2B. CD4+ T cell responses to TB antigens in positive donors. Each donor's cells were placed in six different antigens, with one having no antigens as a negative control, shown on the x-axis. The antigens used were TB at two different concentrations, PPD, TB Lysate, and SEB. The x-axis also shows the four different types of cell expression that were measured; pure CD154+ cells, cytokine-producing cells, all CD154+ cells that did and did not produce cytokines, and cells that were CD154+ and produced cytokines. The four cell types have been shown for the six different environments in which the cells were incubated. The percentage of cells and types of cells that were activated by each of the individual antigens is shown.

Donor #	# cells before CD154+ purification	# cells after CD154+ purification	Purity of CD154+ cells (%)	CD154+ cells from flow cytometry (%)
CF38	8.82×10^6	4×10^5	4.35	61
CF32	5.68×10^6	3.2×10^5	5.63	56

Table 2. Yield and purity of CD154+ CD4+ T cells.

increase their elimination and could be a partial explanation for the increased active TB disease in HIV infected individuals. A better understanding of how to induce CD154 expression on live MTB specific cells will allow physical isolation of CD154 expressing MTB specific cells in HIV+ individuals to test if there actually is increased HIV infection in these cells over the total memory CD4+ T Cell population. This information could have ramifications concerning the recommendation of treating HIV+ individuals in anti-retroviral therapy before or during anti-TB therapy. The results of these studies can be utilized to help understand mechanisms of evasion of host immunity by microbes such as *M. tuberculosis*. A better understanding of the mechanisms of immune evasion would help in developing optimal vaccines.

REFERENCES

1. Kochi, A. 1991. The global tuberculosis situation and the new control strategy of the World Health Organization. *Tubercle 72:1*.

2. Comstock, G. W. 1982. Epidemiology of tuberculosis. *Am Rev Respir Dis 125:8*.

3. Barnes, P. F., A. B. Bloch, P. T. Davidson, and D. E. Snider, Jr. 1991. Tuberculosis in patients with human immunodeficiency virus infection. *N Engl J Med 324:1644*.

4. Chattopadhyay, P. K., J. Yu, and M. Roederer. 2005. A live-cell assay to detect antigen-specific CD4+ T cells with diverse cytokine profiles. *Nat Med 11:1113*.

5. Chattopadhyay, P. K., J. Yu, and M. Roederer. 2006. Live-cell assay to detect antigen-specific CD4+ T-cell responses by CD154 expression. *Nat Protoc 1:1*.

6. Frentsch, M., O. Arbach, D. Kirchhoff, B. Moewes, M. Worm, M. Rothe, A. Scheffold, and A. Thiel. 2005. Direct access to CD4+ T cells specific for defined antigens according to CD154 expression. *Nat Med 11:1118*.

Polymerization of Methyl Methacrylatein Supercritical Carbon Dioxide: A Review

-Matthew Steele Battaglia -

Matthew Battaglia is a Biomedical Engineering student focusing on the Tissue Engineering sequence. He is currently involved in Phi Delta Epsilon's service committee, and was recently inducted member of Tau Beta Pi National Engineering Honors Fraternity. In Matthew's free time, he also volunteers at Rainbow Babies and Children's Hospital. For the past year, he was involved with cystic fibrosis research at the Willard A. Bernbaum Cystic Fibrosis Research Center under current Dean Dr. Pamela Bowes Davis. After graduating from Case Western Reserve University, he hopes to go on to medical school.

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ABSTRACT

Polymers and their processing are a very important part of today's world and the building block of many different industries including computer, automotive, etc. The typical industrial and laboratorial methods of processing and synthesis of these polymers have brought many environmental and cost concerns to the forefront. These concerns have brought about new types of methods of polymerization within recent years, and one such method is polymerization using supercritical fluids (SCFs) such as carbon dioxide. Polymerization using solvents such as supercritical carbon dioxide ($scCO_2$) have helped with the development of many polymers including polymethyl methacrylate (PMMA).

INTRODUCTION

1.1 Polymethyl methacrylate

Polymethyl methacrylate, or PMMA, is a polymer which is synthesized from methyl methacrylate. A representative diagram of PMMA is shown in figure 1.

Isotactic PMMA comprising molecules having a plane of symmetry.

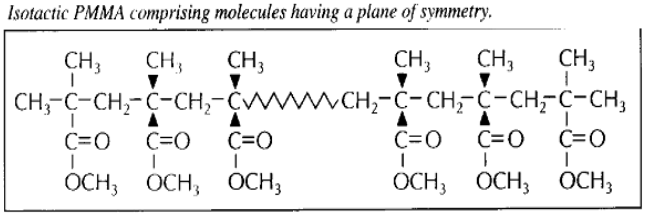


Figure 1 taken from reference Hatada.[1]

It is a strong, rigid, transparent, and colorless amorphous plastic. It has a higher softening point, better weatherability, lower water absorption, good electric resistivity, and a much better impact strength than other polymers such as polystyrene[2, 3]. These characteristics of weatherability and optical clarity, along with lack of color, make PMMA an excellent tool for applications involving light transmission[3]. Examples of general applications include glazing, use in lighting diffusers, skylights, outdoor signs, and exterior lighting lenses in cars and trucks [2].

Other uses of PMMA included lithography, and PMMA is usually the typical resist film used. It has actually been the normal resist in lithography for the past twenty plus years and typically has the highest resolution available[4]. Lithograph techniques create a nanoimprinted design on a thin polymer film such as PMMA which is attached to a substrate. This is outlined in figure 2.

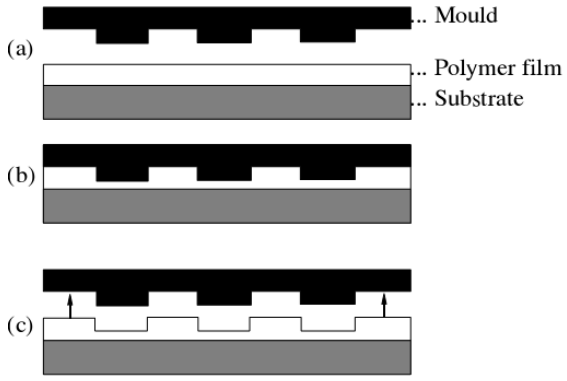


Figure 2 taken from reference Arakcheeva *et al*.[5]

(a) Mould with polymer film substrate,
(b) imprinting process,
(c) mould detachment from polymer

The properties of PMMA such as its low thermal expansion coefficient of around 5×10^5/ ^oC and its low compression ration under pressure, around $5.5 \times 10^{-11} Pa^{-1}$, allow it to be such an excellent resist in nanoprint lithography[5]. The ability of PMMA to act as such a valuable resists assists in the production of commercial production of optical integrated circuits on a photonic crystal basis, as well as the production of semiconductor nanostructures, and many other optical applications such as microgratings for acoustic-optics, integrated optics, spectroscopy, optical interconnects, binary optics, and quantum electronics[6].

PMMA's characteristics allow it to also act as useful in the field of optics. Such an example is the use of $Er(DBM)_3Phen$-doped PMMA which has been very promising in the use of rare-earth doped polymer fibers and waveguides which are instrumental in optical communication stems and sensors, such as local networks and data communications[7]. Just the same, PMMA can act as a luminescent agent when it is grafted to rare earth composite luminescent materials. It has been shown that such things have resistance to water and also produce just as much luminescence as non-PMMA grafted materials[8].

Lastly, PMMA is a material that has had wide biomedical impacts in recent years. This includes the application to drug delivery systems and it is acting as a component of contact lenses. It has been applied in food technology and quality control systems, as well as synthetic membranes for biosensors. The list can go on from there. The application of PMMA in contact lenses is a very great contribution to society and industry as plastics such as this are light in weight, can be mass produced, and are cheaper than their counterpart, glasses[9].

Some common properties that have been tested empirically are listed above in table 1.

Typical Properties of Poly(methyl methacrylate)

Property	PMMA
Density (mg/m^3)	1.18–1.19
Tensile modulus (GPa)	3.10
Tensile strength (MPa)	72
Elongation at break (%)	5
Notched Izod (kJ/m)	0.4
Heat deflection temperature at 1.81 MPa (°C)	96
Continuous service temperature (°C)	88
Hardness (Rockwell)	M90–M100
Linear thermal expansion (10^{-5} mm/mm·K)	6.3
Linear mold shrinkage (in./in.)	0.002–0.008

Table 1: Taken from reference Kutz.[2]

1.2 Supercritical carbon dioxide as a solvent in polymerization

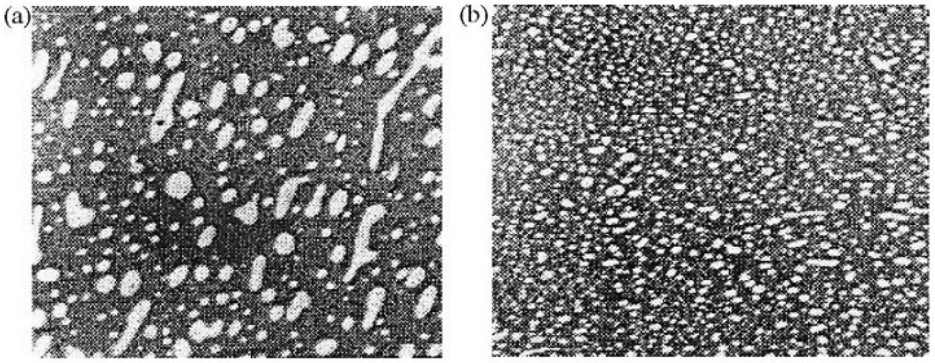
Over the past decades, new processing and methods of polymerization have been created and experimented with in order to reduce problems created by traditional polymerization. The problems include the use of costly, hazardous, flammable, and toxic waste, as well as the generation of large volumes of this aqueous waste. This means nearly 7.000 million metric tons of solvent is used each year, most of which becomes a waste bi-product of the reactions they are used for[3]. Thus, the use of supercritical fluids such as carbon dioxide ($scCO_2$) as a solvent can eliminate many of these problems as it does not have these effects. Other super critical fluids exist, yet super critical carbon dioxide is one of the most studied and easily used.

Aside from the environmental positives, supercritical carbon dioxide is great for carrying out polymerization reactions because it is also economically effective with low costs, the product can be nearly solvent free through simple venting, there is no energy intensive drying and devolatization processes, no chain transfer with free radical polymerizations, as well as the fact that its critical point (T_c = 31.1°C, P_c = 73.8 bar) can easily be attained. Normally, it also dissolves relatively small molecular weight polymers like hydrocarbon solvents. Though it is not especially good at dissolving large molecular weight polymers, stabilizing compounds can be used in dispersion type polymerization reactions in order to synthesize the desired polymer. The surfactants either have a CO_2-philic section to react with the carbon dioxide, or a CO_2-phobic section that reacts with the polymer being made. Along with dispersion reactions, these surfactants help to stabilize the reaction and formation of high molecular weight polymers[10].

Along with these high advantages to using supercritical carbon dioxide as a solvent, reactions using supercritical carbon dioxide also tend to have properties of their own. The use of $scCO_2$ tends to lowers glass transition temperatures of the polymer which creates plasticization of this polymer. This effect allows the removal of residual monomer, incorporation of additives, and also the formation of foams. Also, molecular weight distributions can easily be controlled, as well as unreacted monomer can be regained from the system. Depending on the set up of the system and any stabilizing surfactants and copolymers used, particle size and shape can be controlled, as well as the conversion of a greater amount of monomer to polymer compared to normal synthetic methods[12]. Likewise, this shape and size control element allows for the production of highly porous materials[3].

A developing application of supercritical carbon dioxide as a solvent is its use with blending polymers. This is when two immiscible polymers are mixed to-

gether to form a material with new properties such as impact strength or rigidity. The use of supercritical carbon dioxide has improved the blending process as the scientist can now control the size and relative shape or the components. When blending occurs, usually polymer A and polymer B are mixed, and two phases are formed, one rich in droplets contains polymer B and the other is continuous and rich in polymer A. The visocosity ratio of B to A controls the size of the droplets, and without supercritical carbon dioxide, the shape and size of droplets is relatively uncontrolled. The use of $scCO_2$ allows the scientist to then control the size of these droplets and make a better structured blend of polymers[11]. This is seen in figured 3.



TEM micrographs of PMMA/PS (25/75) blend: (a) without CO_2; (b) with supercritical CO_2. Reproduced from Elkovitch, Lee and Tomasko by permission of the Society of Plastics Engineers, USA.

Figure 3 taken from Nalawade *et al*.[11]

The advantages to using super critical carbon dioxide as a solvent are great. Not only can one modify the solvent for a specific purpose by changing the pressure, but these changes have also allowed for the development of polymers with specific sizes and morphologies. Also, the use of this solvent is also applicable to a wide range or polymerization reactions, including all types of chain and step-growth polymerizations such as ionic, free-radical, and metal-catalyzed reactions. Also, with the development of surfactants, supercritical carbon dioxide is no linger limited to specific polymers, and can produce large, high weight molecular polymers.

This paper will specifically review those different methods involved in the polymerization of high molecular weight MMA in the solvent supercritical carbon dioxide, and how the challenges of its polymerization are met. The focus will be on dispersion polymerizations and the use of surfactants, though there will also be focuses on alternative methods currently being explored, some of which may eliminate the use of stabilizers used in dispersion polymerization.

DISCUSSION

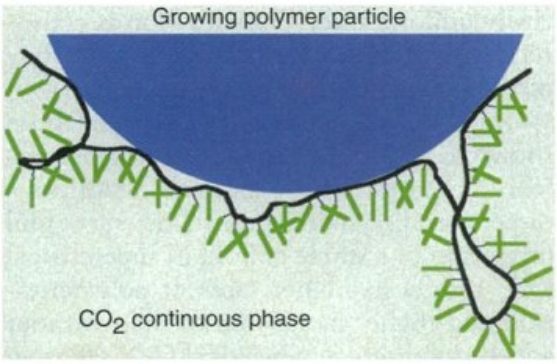
2.1 Dispersion polymerization and the use of surfactants

As previously mentioned, the one problem with using super critical solvents such as carbon dioxide is that larger molecular weight polymers such as polymethyl methacrylate are not soluble in this super critical solvents. A method thoroughly developed and researched by such scientists as DeSimone has found that dispersion polymerization is a key method to facilitating the polymerization of methyl methacrylate in super critical carbon dioxide, though other methods can be somewhat successful.

Dispersion polymerization is defined to be the heterogeneous polymerization process based on the formation of latex particles, usually in the form of a fine powder, in the presence of a stabilizing dispersant

known as the surfactant[10]. The role of this surfactant is to adsorb and chemically attach to the surface of the growing polymer particle. The surfactant will then prevent the particles from aggregating by electrostatic, electrosteric, or steric mechanisms. In super critical CO_2-based systems, the surfactants provide steric stabilization because the carbon dioxide solvent has a rather low dielectric constant. The stabilizing particle tends to be a macromolecule that exists directly at the polymer-solvent interface. It prevents aggregation by coating the surface of the particles and forming long-range repulsions between them. These imparted repulsions must be greater than the attractive force of the existing van der Waals forces existing between the particles[12].

The surfactant can attach to a polymer in a multitude of ways. This includes either chemical grafting or physical adsorption. Physical adsorption is best achieved by using amphiphilic materials (ones that are both hydrophilic and hydrophobic) such as graft or block copolymers. These materials work so well because they have one component in the continuous phase, while the other component is in the polymer phase. The second route to stabilization occurs through chemical grafting of the stabilizer to the polymer particle either through chain transfer or by using such a stabilizer that acts as an initiator, monomer, or terminating agent in the reaction. These chemically grafted stabilizers provide far better colloidal stability than their physical stabilizer counterpart. Typical types of stabilizers used as surfactants include CO_2-philic polymers, block or graft copolymers with both CO_2-philic and CO_2-phobic component, or CO_2-philic reactive macromonomers[12]. An example of how the surfactants interact with particles is shown in Figure 4 where PMMA is interacting with the surfactant PFOA[13].

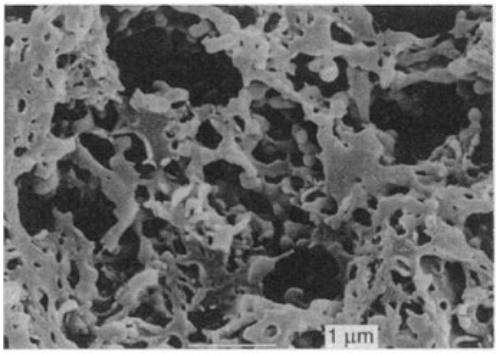


Schematic illustration of a PMMA particle stabilized by poly(FOA) in which the lipophilic backbone acts as an anchor for the fluorocarbon stericstabilizing moieties.

Figure 4 taken from reference DeSimone *et al*.[13]

2.2.1 Poly(1,1-dihdroperfluoro-octylacrylate)

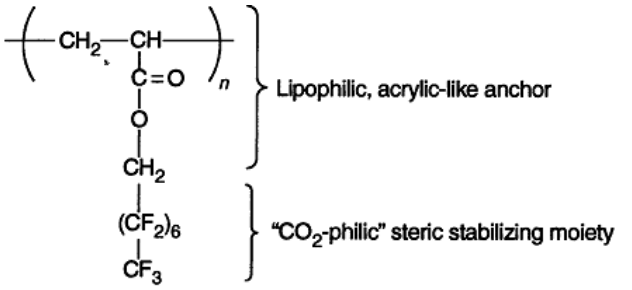
As stated before, high molecular weight polymers such PMMA cannot be formed in super critical carbon dioxides solvent without a stabilizer. PMMA produced without stabilizers tend to form polymers that form thick irregular films on the interior walls of the reaction cells denoting a precipitation reaction with very low conversions and an unstable, unrecognizable morphology[13]. Conversion percentages of less than 40% were observed[12]. This unrecognizable morphology is depicted in figure 5.



Scanning electron micrograph of PMMA synthesized in CO_2 without stabilizer.

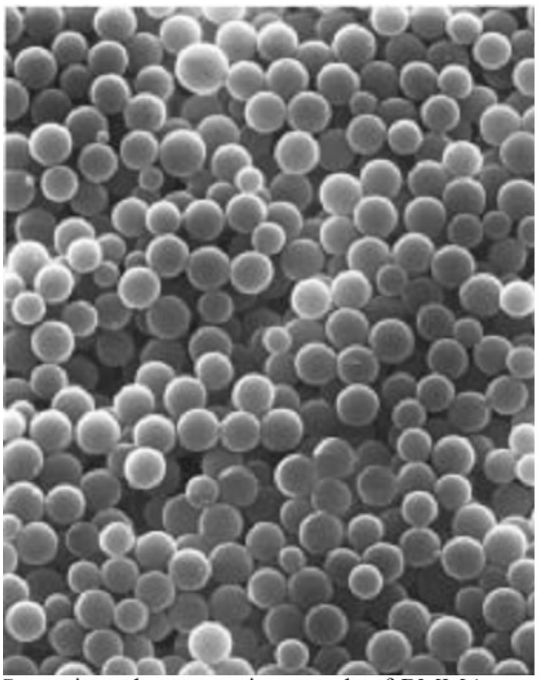
Figure 5 taken from reference DeSimone *et al*.[13]

In 1994, the novel approach to add stabilizers to the dispersion polymerization reaction was used by DeSimone and his team. They employed the use of poly (1,1-dihdroperfluorooctylacrylate) or PFOA pictured in figure 6[12]. The amphiphilic nature of this homopolymer reacted well with the lipophilic monomer. The reaction ended up polymerizing MMA to high conversion rates of greater than 90% and high degrees of polymerization as well (greater than 3000) in the supercritical solvent. The experiments were conducted at 65°C and a pressure of 207 bar with AIBN or fluorinated derivative of AIBN. Not only did the polymer produced have high conversion percentage, but it formed relatively even spherical particles that had a narrow size range of one to three micrometers[12]. DeSimone also found that increasing the dispersant concentration led to much finer, smaller and more uniform particles. Also increasing the overall molecular weight of the stabilizer resulted in a greater increase in particle size[14]. The new, finely made product is pictured in figure 7. The ending results were also significant as it was found that 83% of the solvent could be removed after drying and depressurizing of the PMMA from the carbon dioxide occurred. This is very significant as the stabilizer is of high cost, and thus can be recycled for use in following experiments[12].



The chemical structures of an interfacially active, polymeric stabilizer [poly(FOA)], depicting the proposed site of anchorage for the CO_2-philic steric stabilizing moieties.

Figure 6 taken from reference Kendall *et al.*[12]



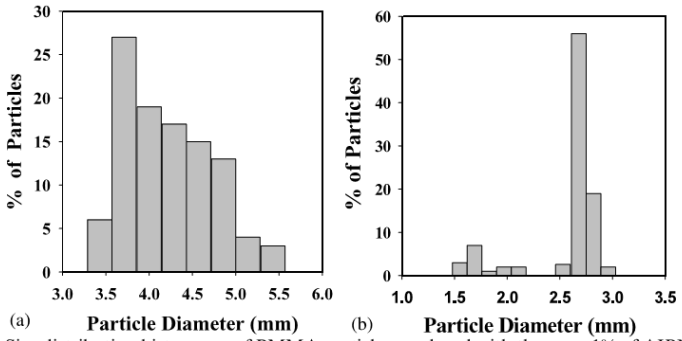
Scanning electron micrograph of PMMA particles produced by dispersion polymerization in CO_2 using PFOA as the stabilizer.

Figure 7 taken from reference Kendall *et al.*[12]

2.2.2 Polydimethylsiloxane

Another more recently studied stabilizer is polydimethylsiloxane (PDMS). This stabilizer has been sought after because of the relative expensive nature of fluorinated polymers[12]. Despite the advantage of being cheaper, PDMS-based surfactants are less soluble in $scCO_2$ because monomer is continuously needed in the continuous phase, and thus these surfactants generally have to be capped with some sort of methacrylate in order to help solvate the PDMS tails and prevent flocculation, which is the formation of polymers attaching to each other, forming a fragile structure[12,15]. Other times, the PDMS has other attachments.

Countless numbers of different forms of the stabilizers have been tested and different ones have worked at different conditions. One such example of a derivative of PDMS is PDMS-g-pyrrolidone carboxylic acid (Monasil PCA), which is a CO_2-philic stabilizer. Using this stabilizer, a 94% conversion was achieved at supercritical conditions of 338.2 K and 345 bar. Unfortunately though, this method is not perfect. It was found that when the polymer was not well stabilized the conversions were below 90%, and the polymer formed was heavily agglomerated, forming a cake-like morphology. Despite this, shape of stable particles formed were of average size, spherical in shape, and the average size decreased exponentially with increase the amount of surfactant. Also, the issue of agglomeration was solved with an increase in the amount of surfactant used. As surfactant concentration was increased, the particle size distribution narrowed, and it allowed for a more uniform polymer. This is very important as it allows for a product that is usable in both industry and the laboratory settings. Figure 8 accounts for statements presented earlier about the size distribution range seen at a lower and higher concentration of the stabilizer. Stable, acceptable PMMA was polymerized when at least 7% of the Monasil PCA was used[16].



Size distribution histograms of PMMA particles produced with the same 1% of AIBN but with different amount of CO_2-philic surfactant. (a) 6.1 wt.% and (b) 15.1 wt.% of the MMA.

Figure 8 – Taken from Reference Park and Shim.[16]

Another very popular PDMS derivative that has been used in the polymerization of MMA has been polydimethylsiloxane-methyl methacrylate of PDMS-mMA macromonomer. Like other forms of PDMS, both a minimum pressure, as well as a stabilizer concentration has to be reached in order to reduce particle coagulation and prevent steric stabilization. This pressure is 3000 psia and around a 2 weight % of stabilizer to monomer. Again, greater concentrations of the macromonomer lead to a general greater percent yield of the PMMA product and also smaller, finer particles[15].

These types of surfactants obviously have their advantages and disadvantages. Unlike PFOA and its derivatives, PDMS derivatives are much cheaper because they do not use fluorine associated molecules. Despite this advantage, the decreased solubility of the surfactant in the solution means PDMS cannot be used, and multitudes of derivatives have to be formed in order for the dispersion polymerization to occur and form

PMMA. In fact, it seems direct conditions such as specific pressure settings, and also much higher weight percentages of the stabilizer to monomer have to be used. Also, reaction time also plays a factor; as reaction time increases, coagulation is less frequent and stable latex particles are formed[15]. This is seen in table 2.

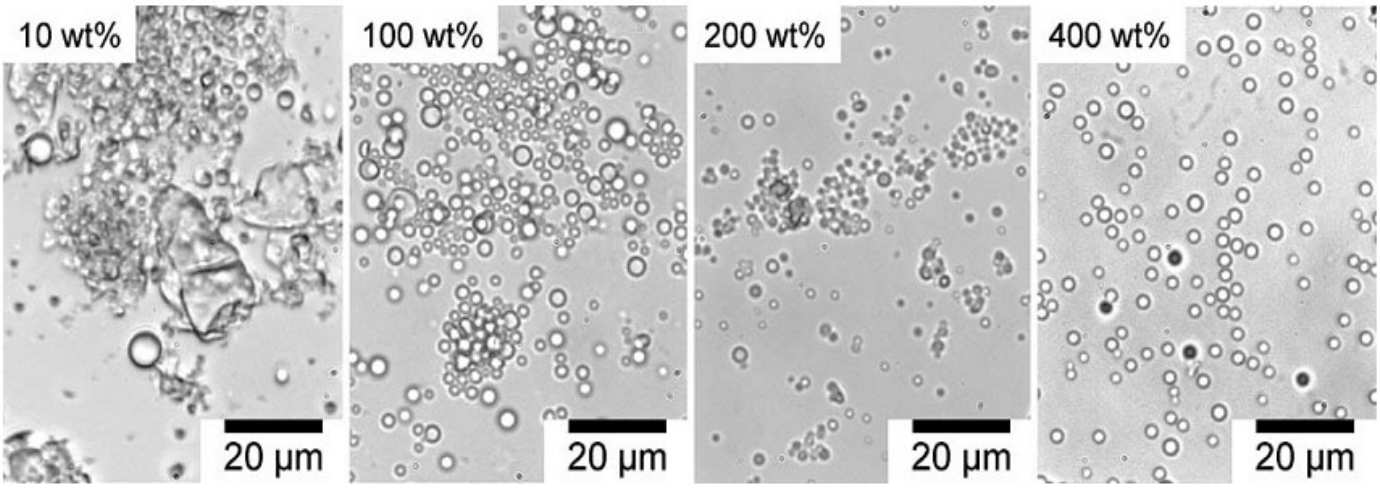
If proper conditions are not met, coagulation and agglomeration usually occur and form a product with low percent yield and a morphology that cannot be used in any real sense. An excellent example is the use of another derivative of PDMS known as trimethylsiloxy terminated PDMS (TS-PDMS). As the weight percentages of the stabilizer are increased as compared to the monomer, the morphology changes becoming much finer and the particle size decreases to a useable PMMA product[17]. This is clearly shown in figure 9.

Dispersion Polymerization of MMA in CO_2 at Various Pressures and Times[a]

time (h)	pressure (psia)	yield (%)	M_w ($\times 10^3$g/mol) /PDI	product morphology	approximate particle size (μm)/PDI
5	4000	88	365/3.9	uncoagulated	3.3/1.08
2	4000	68	247/4.0	n/a	n/a
1	4000	15	n/a	n/a	n/a
5	3000	93	339/4.0	partially coagulated	3.1/1.11
5	1500	90	481/4.5	coagulated	2.5/1.28
2	2000	34	203/10.9	n/a	n/a
1.25	2000	12	n/a	n/a	n/a

[a] All systems were 25−30 wt % MMA/CO_2, ~5 wt % PDMS-mMA/MMA, ~1 wt % AIBN/MMA, and 65 °C.

Table 2 taken from reference O'Neill *et al*.[15]



Optical micrographs of *n*-hexane redispersions of centrifugally washed PMMA particles with *n*-hexane, which were prepared by dispersion polymerizations with BPO (4.0 mmol/L) at different TS-PDMS concentrations (wt% based on MMA) in $scCO_2$

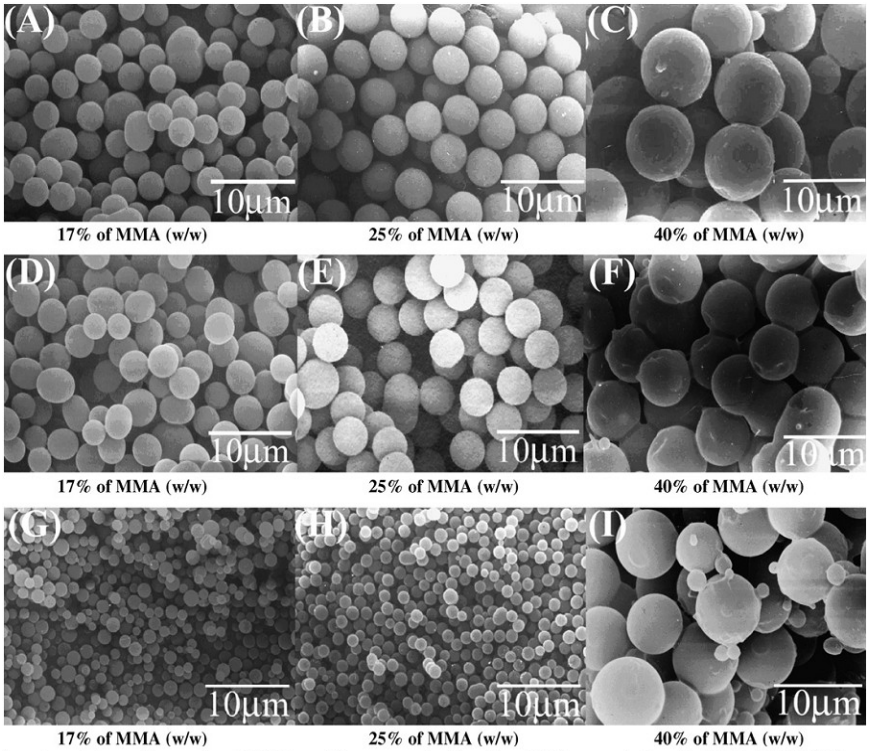
Figure 9 – Taken from reference Fujii *et al*.[17]

2.2.3 Random Copolymers

Though block and graft copolymers containing siloxane or fluorinated polymers such as PDMS and PFOA derivatives have been very successful in the laboratory setting when synthesizing PMMA, the small limitations to these methods have had scientists query further into the dispersion polymerization of PMMA. Fluorpolymer stabilizers are so expensive and most other graft and block copolymers such as PDMS derivatives are complex and difficult to synthesize, and they also agglomerate when conditions are not perfect[18]. Also, these type of block and graft polymers cannot be characterized in usually solvents because of their poor solubility[19]. In order to solve these problems, scientists have tested the development and use of random copolymers containing small amounts of fluorine to act as the soluble part and were incorporated with a comonomer of MMA which acted as the binding part to the MMA particles[18].

One such experiment used three semi-fluorinated random copolymers using fluorinated octylmethacrylate (FOMA) and three different comonomers of methacrylate derivatives, including n-butyl methacrylate (BMA), 2-dimethylaminoethyl methacrylate (DMAEMA), and methyl methacrylate (MMA). The three random copolymers used were poly(FOMA-co-DMAEMA), poly(FOMA-co-MMA), and poly(FOMA-co-BMA). Each of these stabilize were used in supercritical carbon dioxide where the temperature was 65^{o}C, a pressure of 345 bar, a reaction time of 12 hours, 2% stabilizer, and 1% of the initiator AIBN. As the reaction concluded, it was shown that only the poly(FOMA-co-DMAEMA) surfactant produced good latex stability of the PMMA and that the particles were fine with a relatively uniform size, while the other stabilizers formed flocculated, white clumpy solids that were not usable. Thus, not all random copolymers are usable to produce stable PMMA particles, but a result that was surprising was that a poly(FOMA-co-DMAEMA) of just 34 w/w% of the FOMA was used, as compared to previous experiments which suggested that much higher weight percentages of the fluorinated polymers have to be used in the polymer in order to have a stable polymerization with high conversion percentages[18].

Another experiment involved the use of poly (poly(ethylene glycol) methacrylate-co-1H,1H,2H,2H-perfluorooctylmethacrylate) or p(PEGMA-co-FOMA). This experiment incorporated the same tests as other experiments, but another characteristic put into question was the effect of the pendent chain length of the PEGMA copolymer. The stabilizers containing greater amounts of the FOMA, or fluorinated copolymer, were found to have higher conversions of PMMA as well as small, finer powders of the PMMA. The lower concentrations of FOMA in the copolymers did produce very high conversion, but the product formed somewhat irregular shapes of particles as well also small amounts of agglomerated product was formed, though it would easily be broken up. Secondly, the stabilizers containing larger pendant chain lengths of the PEGMA formed a finer powder instead of a clumpy solid. It seemed that the extended length of 1100 allowed for a greater anchoring of the PMMA particles and prevented particle aggregation. Furthermore, the smaller chains lengths of 300 and 475 were much more soluble in the supercritical carbon dioxide, while the PEGMA having a length of 1100 was less soluble, and thus able to absorb onto the

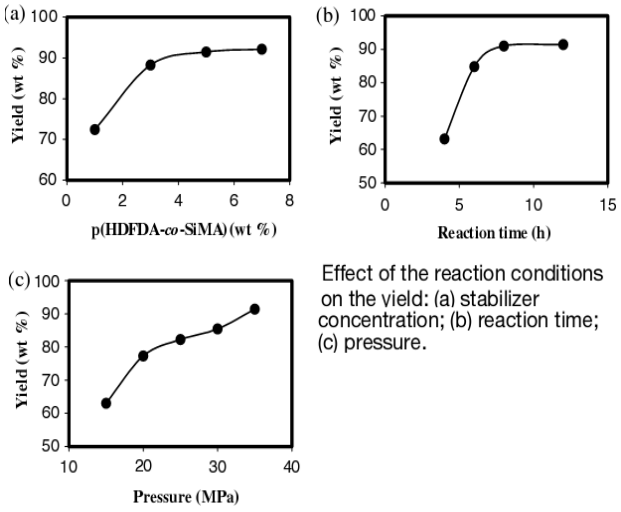


Scanning electron micrographs of PMMA particles prepared with various MMA concentrations (w/v): (A–C) (PEGMA (475)-*co*-FOMA) (33:67), (D–F) p(PEGMA (475)-*co*-FOMA) (16:84), and (G–I) p(PEGMA (1100)-*co*-FOMA) (12:88). Reaction conditions: 5% of stabilizer (w/w to MMA), 65 °C, 345 bar, and reaction time: 12 h.

Figure 10 – Taken from reference Hwang *et al*.[19]

PMMA particles and provide better anchoring. Lastly, this experiment showed that increasing concentration of the monomer, MMA, allowed for greater size of the resulting PMMA. All of this is seen in figure 10, as MMA concentration is increased the size of the PMMA particles increases, as FOMA content is increased, the particles become more spherical in nature, and as the pendant chain length is increased, the particles form finer powders[19].

Like the PDMS derivatives, random copolymers and their efficiency in polymerizing MMA are affected by pressure, concentration of the stabilizer, and reaction time. Like all other random copolymer stabilizers, an experiment conducted using poly((heptadecafluorodecyl acrylate)-co-3-(tris(trimethylsilyloxy)silyl)propyl methacrylate, or p(HDFDA-co-SiMA), as the stabilizer showed that increasing the weight percentage of the stabilizer in the solution increased the yield, number molecular average, decreased the PDI and decreased the diameter of the PMMA product creating a fine, white powder which is usable. Relatively low concentration of the copolymer, just like most reactions, produces an unusable, unrecognizable morphology in solid form. This random copolymer was also tested, as stated before, for reaction time and pressure effects as well on the yield of PMMA[20]. The relationship between these effects and their relationship to the conversion of MMA to PMMA is seen in figure 11.



Effect of the reaction conditions on the yield: (a) stabilizer concentration; (b) reaction time; (c) pressure.

Figure 11 taken from reference Deniz *et al*.[20]

Carole Leppilleur and Eric Beckman state it best when describing the use of random copolymers, such as p(MMA-co-HEMA)-g-PFPO which they tested. Along with pressure and reaction time, there must be a great balance between the soluble part and the anchor group of the stabilizer at hand. If properly done, the balance

should provide proper adsorption of the MMA particle leading to smaller, narrowly dispersed particles with larger molecular weights, and the proper amount of the soluble component will allow for proper stabilization, prevent larger particle sizes and greater size distributions. Of course, like all other methods used in dispersion polymerization of PMMA in supercritical carbon dioxide, concentration of the stabilizer as a whole is also very important[14].

2.3 Catalytic chain transfer used in polymerizations of PMMA utilizing supercritical carbon dioxide

Polymerization in supercritical carbon dioxide has also been used by some scientists to produce small molecular weight and medium molecular weight PMMA using catalytic chain transfer instead of the typical stabilizing agents. This has been employed using transition-metal catalyst tetraphenylcobalt-oxime boron fluoride (COPhBF) as a chain transfer agent[21, 22].

Successful polymerization of medium ranged molecular weight PMMA product was developed using COPhBF in the presence of a PDMS-ma surfactant. It is important to note that this reaction only works with a surfactant, thus employing a dispersion polymerization. Without such a surfactant, the resulting polymer is a sticky solid or a viscous fluid. Otherwise, the use of a surfactant produces a free-flowing white powder with high conversion percentages to the product, PMMA.

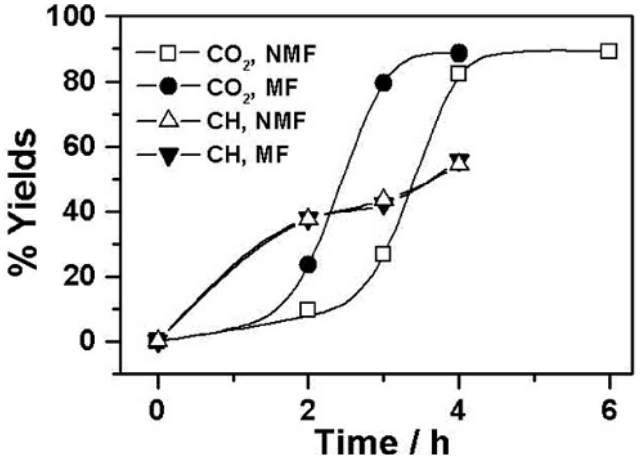
Experimental results also showed that as the concentration of the catalyst was increased, molecular weight continued to decrease, producing polymers with number molecular weights of 20,000 to 50,000 depending on the concentration of the catalyst. The only problem was the ending polymer was partially flocculated in morphology. This flocculation was most likely caused by the supercritical carbon dioxide being able to easily plasticize the polymer because of its small molecular weight. The small molecular weight distributions shown in the experimental results show that the catalytic chain transfer agent's activity was reduced, thus allowing or medium sized polymers in terms of number average molecular weight[21].

Polymerizations using supercritical carbon dioxide and the catalytic transfer agent COPhBF, but not a stabilizing agent, produced PMMA characterized as very small, oligmeric acrylics. Though the molecular weight distributions were much narrower in supercritical carbon dioxide than other tested solvents such as toluene and bulk MMA, but the products were much smaller in number average molecular weight. This proves that the supercritical carbon dioxide solvent acts in helping the chain transfer rate as it is greatly increased, thus producing smaller polymers. As originally stated, supercritical carbon dioxide allows for control of the resulting polymer in terms of size and molecular weight. In this case, as opposed to dispersion polymerization, polymerization using a chain transfer agent in this supercritical solvent allows for the formation of a much smaller sized PMMA[22].

2.4 Other methods used to change molecular weight of the PMMA product

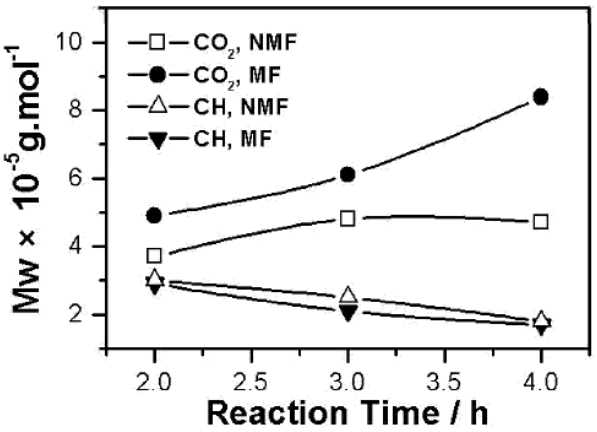
2.4.1 Magnetic fields

A recent study was conducted where magnetic fields were tested in polymerizing MMA in different solvents, including cyclohexane and supercritical carbon dioxide. Though a dispersant, or surfactant, was not used, the implication was that the resulting effects of the study could also be employed with dispersion reactions of MMA in supercritical carbon dioxide. The authors used a 3000 G external magnetic field when testing the magnetic field effect on the polymerization of MMA. The results revealed that the magnetic field effect increased the weight average molecular weights of the PMMA, and that the presence of a magnetic field produced a higher molecular weight polymer than without. The experiment also showed that at a certain time in the polymerization, percent yield was actually three times larger than the PMMA polymer produced without the magnetic field. The polymerization of MMA in the cyclohexane solvent was not affected by the magnetic field at all. These results are summarized in figures 12 and 13. The authors determined that the magnetic field actually accelerated the precipitate phase, which is significant because swelling of the PMMA caused by supercritical carbon dioxide allowed for more of the PMMA to be in the precipitate phase[23].



The yield of PMMA at different reaction times in SC CO_2 and cyclohexane with and without MF.

Figure 12 taken from reference Liu *et al.*[23]



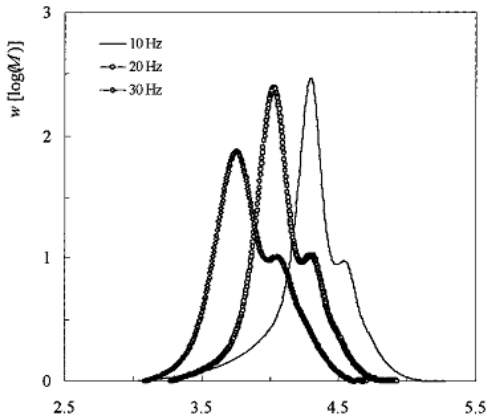
The weight average molecular weight of PMMA at different reaction times in SC CO_2 and cyclohexane with and without MF.

Figure 13 taken from reference Liu *et al.*[23]

2.4.2 Pulsed lasers vary molecular weight distribution

Pulsed-laser polymerization, or PLP, works by using laser pulses to create radical from a photointiator. Though these radicals may combine bimolecularly, the instantaneous and large increase in radical concentration allows the growth of many new polymer chains. Just like the use of magnetic fields, this use of PLP has not been tested using surfactants, but has been used in supercritical carbon dioxide solvents with MMA. The photointiator makes it so no initiator is needed in the

reaction. Upon different laser repetition frequencies, the molecular weight of PMMA was changed. As the frequency of the firing increased, molecular weight decreased as more and more radicals were formed, breaking the existing polymeric chains. Laser energy and photoinitiator concentration did not seem to effect the reaction [24]. This is seen in figure 14. Though this technique does not employ the use of surfactants and dispersion polymerization, it can be a useful method in the future because it can control and change the length and size of PMMA chains based on firing frequencies of the laser.



Molecular weight distribution (MWD) for PMMA produced in $scCO_2$ by pulsed-laser polymerization at variou laser repetition frequencies: [DMPA] = 6.7 mmol·L^{-1}, [MMA] = 6.2 mol·L^{-1}, T = 25 °C, and P = 180 bar.

Figure 14 taken from Quadir *et al*.[24]

2.5 Future considerations

As with all techniques, many of the conditions and methods used could be improved to make the process and end product much better than they already are. For example, pressure and temperatures settings could be optimized for the supercritical carbon dioxide solvent, monomer, initiator, and surfactant concentrations could be optimized, as well as the development of better surfactants would aid these processes.

With all these possible fine tunings to dispersion polymerization, other methods are being developed for the polymerization of MMA in supercritical carbon dioxide. As has been mentioned, pulsed-laser polymerization, the use of magnetic fields, and catalytic chain transfer agents are just some of the newly developed possibilities in the supercritical solvent. Another such future possibility is the use of ultrasound assisted polymerization.

Ultrasound assisted polymerization works by using wave frequencies ranging from 16kHz to a few megahertz. When an ultrasound of proper amplitude passes through the medium in use, large numbers of microbubbles form, grow, and collapse in a very short time. This cavtiation phenomenon provides the mechanism for the sonochemistry effect. During cavitation, the bubbles collapse and produce intense local heating and high pressures during a short period of time. Cavitation creates temperatures greater than 5000^{o}C and pressures above 1000 atm at cooling rates above 1000 K/s. These extremely harsh conditions allow for the production of excited states which are able to break chemical bonds and form free radicals. Secondly, ultrasound has unique properties which have strong dispersing, stirring, and emulsifying effects which allow for new routes of stabilization of dispersion latex [25].

Though the conducted experiment did not use supercritical carbon dioxide, the authors used near supercritical conditions, including very high pressures. The experiment revealed that after periods of two, four and six hours, the number and weight average molecular weights of PMMA increased. Also, the CO_2/monomer

ratio was also significant. As the monomer concentration was raised, the molecular weight of the ending PMMA chain increased and the molecular weight distribution decreased because the resultant polymer was more soluble in solution. Though the experiment was not conducted at supercritical conditions, the authors hinted this could possibly be used in such conditions because this experiment produced a stable dispersion polymerization. They also noted the advantage that there was no necessity for a stabilizer using ultrasound irradiation in the polymerization of PMMA[25].

CONCLUSION

Modern methods of polymer processing have come into question because of their cost and waste producing drawbacks. The development of supercritical carbon dioxide as a solvent has acted as a monumental solution to these laboratory and industrial related polymer processing problems. Not only is it considered a "green solvent," being safe for the environment, but it is also a much cheaper solvent as it is just carbon dioxide as extreme conditions. The development of such a solvent is very important as it has impacted the processing of polymethyl methacrylate. This new technique thus allows for easier processing of PMMA which is important in many important areas today, such as optics, biomedical, and glass-related products.

Due to some limitations of supercritical carbon dioxide, dispersion polymerization, the use of surfactants as stabilizers, was developed in order to incorporate PMMA into the supercritical solvent. Since the dawn of its use, many different surfactants with different properties have been developed. Scientists are continually trying to improve the process so production of more stable, higher molecular weight latex PMMA particles will be created. Other methods have been attempted, some of which employ the idea that stabilizers may not be needed. These methods include the use of catalytic chain transfer, and the possibilities of PLP, magnetic fields, and ultrasound in this supercritical solvent. Continued research and development will eventually lead to a method utilizing supercritical carbon dioxide that will produce PMMA with high conversion, very high stability, and high molecular weight.

REFERENCES

1. K. Hatada, "Uniform Poly(methyl methacrylate)s with Controlled Architecture," *TRIP*, **5**, 223-229, (1997).
2. M. Kutz,"Handbook of Materials Selection," J. Wiley, New York, (2002) pp. 341.
3. N. Ajzenberg, F. Trabelsi, and F.,Recasens, "What's New in Industrial Polymerization with Supercritical Solvents? A Short Review," *Chem. Eng. Technol.*, **23**, 829-839, (2000).
4. E.A. Dobisz, S.L. Brandow, R. Bass, and J. Mitterender, "Effects of molecular properties on nanolithography in polymethyl methacrylate," *J. Vac. Sci. Technol B.,* **18**, 107-111, (2000).
5. E.M. Arakcheeva, E.M. Tanklevskaya, S.I. Nesterov, M.V. Maksimov, S.A. Gurevich, J. Seekamp, and C.M. Sotomayor Torres, "Fabrication of Semiconductor- and Polymer-Based Photonic Crystals Using Nanoimprint Lithography," *Technical Physics*, **50**, 1043-1047, (2005).
6. Y. Li and S. Sugiyama, "Fabrication of micrograting on PMMA plate and curved surface by using copper mesh as X-ray lithography mask," *Microsyst. Technol.*, **13**, 227-230, (2007).
7. Z. Zheng, H. Liang, H. Ming, Q. Zhang, and J. Xie, "Optical transition probability of the Er^{3+} ion in $Er(DBM)_3$phen-doped poly(methyl methacrylate)," *Opt. Commun.*, **233**, 149-153, (2004).
8. L. Peng, Y. Luo, Y. Dan, L. Zhang, Q. Zhang, S. Xia, and X. Zhang, "The study of preparation and luminescence of polymethyl methacrylate/rare earth composite luminescent materials," *Colloid Polym. Sci.*, **285**, 153-160, (2006).
9. C. Lee, J. Hsu, and C. Jaing, "Optical coatings on polymethyl methacrylate and polycarbonate," *Thin Sol. Films*, **295**, 122-124, (1997).
10. S. Fortini, F. Lavanchy, P. Nising, and T. Meyer, "A new tool for the study of polymerization under supercritical conditions – preliminary results," *Macromol. Symp.*, **206**, 79-92, (2004).
11. S.P. Nalawade, F. Picchioni, and L. Janssen, "Supercritical carbon dioxide as a green solvent for processing polymer melts: Processing aspects and applications," *Prog. Polym. Sci.*, **31**, 19-43, (2006)
12. J.L Kendall, D.A Canelas, J.L. Young, and J.M. DeSimone, "Polymerizations in Supercritical Carbon Dioxide," *Chem. Rev.*, **99**, 543-563, (1999).
13. J.M. DeSimone, E.E. Maury, Y.Z. Meceloglu, J.B. McClain, T.J. Romack, and J.R. Combes, "Dispersion Polymerizations in Supercritical Carbon Dioxide," *Science*, **265**, 356-359, (1994).
14. C. Lepilleur and J. Beckman, "Dispersion Polymerization of Methyl Methacrylate in Supercritical CO_2," *Macromolecules*, **30**, 745-756, (1997).
15. M.L. O'Neill, M.Z. Yates, K.P. Johnston, C.D. Smith, and S.P. Wilkinson, "Dispersion Polymerization in Supercritical $CO_2$with a Siloxane-Based Macromonomer: 1. The Particle Growth Regime," *Macromolecules*, **31**, 2838-2847, (1998).
16. J. Park and J. Shim, "Emulsion stability of PMMA particles formed by dispersion polymerization of methyl methacrylate in supercritical carbon dioxide," *J. Supercrit. Fluids*, **27**, 297 – 307, (2003).

17. S. Fujii, H. Minami, and M. Okubo, "Preparation of poly(methyl methacrylate) particles by dispersion polymerization with organic peroxide in the presence of trimethylsiloxy terminated poly(dimethylsiloxane) in supercritical carbon dioxide. *Colloid Polym. Sci.*, **284**, 327-333, (2005).
18. H.S. Hwang, Y. Gal, K.P. Johnston, and K.T. Lim, "Dispersion Polymerization of Methyl Methacrylate in Supercritical Carbon Dioxide in the Presence of Random Copolymers," *Macromol. Rapid Commun.*, **27**, 121-125, (2006).
19. H.S. Hwang, W. Lee, S. Hong, S. Jin, and K.T. Lim, "Dispersion polymerization of MMA in supercritical CO_2 in the presence of poly(poly(ethylene glycol) methacrylate-co-1H,1H,2H,2H-perfluorooctylmethacrylate)," *J. Supercrit. Fluids*, **39**, 409-415, (2007).
20. S. Deniz, N. Baran, M. Akgün, I.N. Uzun, and S. Dinçer, "Dispersion polymerization of methyl methacrylate in supercritical carbon dioxide using a silicone-containing fluoroacrylate stabilizer," *Polym. Int.*, **54**, 1660-1668, (2005).
21. W. Wang, D.J. Irvine, and S.M. Howdle, "Dispersion Catalytic Chain Transfer Polymerizations of Methyl Methacrylate in Supercritical Carbon Dioxide," *Ind. Eng. Chem. Res.*, **44**, 8654-8658, (2005).
22. D.J. Forster, J.P.A. Heuts, F.P Lucien, and T.P. Davis, "Catalytic Chain Transfer Polymerization of Methyl Methacrylate in Supercritical Carbon Dioxide: Evidence for a Diffusion-Controlled Transfer Process," *Macromolecules*, **32**, 5514-5518, (1999).
23. J. Liu, R. Zhang, H. Li, B. Han, Z. Liu, T. Jiang, J. He, Z. Zhang, and G. Yang, "How does magnetic field affect polymerization in supercritical fluids? Study of radical polymerization in supercritical CO_2," *New J. Chem.*, **26**, 958-961, (2002).
24. M.A. Quadir, J.M. DeSimone, A.M. v. Herk, and A.L. German, "Pulsed-Laser Polymerization of Methyl Methacrylate in Liquid and Supercritical Carbon Dioxide," *Macromolecules*, **31**, 6481-6485, (1998).
25. R. Wang and M. Cheung, "Ultrasound assisted polymerization of MMA and styrene in near critical CO_2," *J. Supercrit. Fluids*, **33**, 269-274, (2005).

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