A Novel Ultrashort Pulsed Laser Method for Pathogen Inactivation

Shaw-Wei David Tsen
Washington University in St. Louis

Follow this and additional works at: http://openscholarship.wustl.edu/art_sci_etds

Part of the Molecular Biology Commons

Recommended Citation
http://openscholarship.wustl.edu/art_sci_etds/780

This Dissertation is brought to you for free and open access by the Arts & Sciences at Washington University Open Scholarship. It has been accepted for inclusion in Arts & Sciences Electronic Theses and Dissertations by an authorized administrator of Washington University Open Scholarship. For more information, please contact digital@wumail.wustl.edu.
A Novel Ultrashort Pulsed Laser Method for Pathogen Inactivation

by

Shaw-Wei David Tsen

A dissertation presented to the
Graduate School of Arts and Sciences
of Washington University in
partial fulfillment of the
requirements for the degree
of Doctor of Philosophy

May 2016

St. Louis, Missouri
Table of contents

List of figures.................................................................................................................. iii
List of tables .................................................................................................................... v
Acknowledgements ........................................................................................................ vi
Abstract of the Dissertation ............................................................................................ vii
Chapter 1: Introduction ..................................................................................................... 1
    References .................................................................................................................. 15

Chapter 2: Inactivation of enveloped virus by laser-driven protein aggregation ............. 19
    References .................................................................................................................. 40

Chapter 3: Studies of the inactivation mechanism of non-enveloped icosahedral viruses by a visible ultrashort pulsed laser ..................................................................................... 45
    References .................................................................................................................. 69

Chapter 4: Ultrashort pulsed laser treatment inactivates viruses by inhibiting viral replication and transcription in the host nucleus ................................................................. 72
    References .................................................................................................................. 91

Chapter 5: Pathogen reduction in human plasma using an ultrashort pulsed laser ........... 93
    References .................................................................................................................. 113

Chapter 6: A novel chemical-free inactivated whole influenza virus vaccine prepared by ultrashort pulsed laser treatment .................................................................................. 115
    References .................................................................................................................. 137

Chapter 7: Conclusions ................................................................................................... 139

List of Graduate Publications ........................................................................................ 142
List of Figures

Chapter 1

Fig 1. Generic structure of an enveloped virus.................................................................3

Fig 2. The concept of pathogen reduction of blood products..............................................8

Fig 3. The concept of virus inactivation for vaccine generation. 11

Fig 4. (a) General schematic diagram of a pulsed laser. (b) The experimental setup for ultrashort pulsed laser treatment.................................................................14

Chapter 2

Fig 1. Experimental setup for femtosecond laser treatment.............................................26

Fig 2. Inactivation of MCMV using a femtosecond laser..................................................30

Fig 3. Preservation of MCMV global virion structure after femtosecond laser treatment.......31

Fig 4. Preservation of MCMV genomic DNA integrity after femtosecond laser treatment......32

Fig 5. Aggregation of MCMV virion proteins after femtosecond laser treatment.................33

Chapter 3

Fig 1. (a) Graphs of plaque forming units (PFU)/ml for control (without laser treatment) and laser-treated MNV-1 purified samples. (b) Graphs of plaque forming units (PFU)/ml for control (without laser treatment) and laser-treated MNV-1 unpurified samples..............................................54

Fig 2. The fraction of survival as a function of laser power density for a MNV-1 sample.......55

Fig 3. TEM images of MNV-1 (a) without laser treatment (the control); (b) with laser treatment at a power density of $1.1 \pm 0.2 \text{ MW/cm}^2$ (here, for the sake of clarity, only one inactivated MNV-1 is shown); (c) with laser treatment at a power density of $100 \pm 10 \text{ MW/cm}^2$........................57

Fig 4. MNV-1 survival as a function of laser spectral width..............................................58

Fig 5. Real-time PCR counts in linear scale for the genome of MNV-1 without (control) and with laser treatment at an average laser power density of 100 MW/cm$^2$.......................59

Fig 6. The MNV-1 survival fraction as a function of laser power density, fit with Eq. (3) for laser power density < 80 $\text{MW/cm}^2$ (solid line).........................................................65
Chapter 4

Fig 1. Proposed model for USP laser-induced protein aggregation .......................... 76
Fig 2. Electron microscopy shows cellular internalization of USP laser-treated MCMV .... 82
Fig 3. Fluorescence microscopy shows cellular internalization of USP laser-treated MCMV .... 82
Fig 4. MCMV DNA is present in cells infected with USP laser-treated MCMV ............... 83
Fig 5. USP laser-treated MCMV cannot replicate in cells ........................................... 84
Fig 6. IE1 protein is absent in cells infected with USP laser-treated MCMV ................. 85
Fig 7. DNA from USP laser-treated MCMV virions is PCR-amplifiable ...................... 86
Fig 8. Proposed model for USP laser-induced viral capsid defect ............................... 88
Supplementary Fig 1. Virus inactivation efficiency .................................................. 90

Chapter 5

Fig 1. Inactivation of viruses in plasma using a USP laser ....................................... 103
Fig 2. SDS-PAGE and native PAGE analysis of control and USP laser-treated plasma proteins. .................................................................................................................................. 106
Fig 3. Structural analysis of control and laser-treated fibrinogen protein .................... 108
Fig 4. Aggregation state of control and laser-treated fibrinogen protein ...................... 111

Chapter 6

Fig 1. Body weight changes in H1N1-challenged mice ............................................. 128
Fig 2. CD8+ T cell induction following vaccination ................................................... 129
Fig 3. Neutralizing antibodies detected by microneutralization assay ....................... 130
Fig 4. Transmission electron microscopy images of influenza virus ......................... 131
Fig 5. Carbonyl content of laser-treated BSA protein .............................................. 132
List of Tables

Chapter 1

Table 1. Killing efficacy for a variety of viruses using a 425 nm femtosecond pulsed laser….12

Chapter 2

Table 1. MCMV proteins from the aggregate identified by LC-MS/MS…………………34

Table 2. Dynamic Light Scattering data for a variety of laser-treated proteins in buffered solution………………………………………………………………………………………………….37

Chapter 5

Table 1. Protein retention for USP laser-treated plasma……………………………………..104

Chapter 6

Table 1. Hemagglutination activity of live and the USP laser-inactivated influenza virus…..126
Acknowledgements

The work in this dissertation was supported in part by the Mallinckrodt Institute of Radiology, and by grants from the National Institutes of Health and from the National Heart, Lung, and Blood Institute.
ABSTRACT OF THE DISSERTATION

A Novel Ultrashort Pulsed Laser Method for Pathogen Inactivation

by

Shaw-Wei David Tsen

Doctor of Philosophy in Biology & Biomedical Sciences

Molecular and Cellular Biology

Washington University in St. Louis, 2016

Professor Samuel Achilefu, Chair

Emerging viral pathogens represent a constant and prominent threat to human health worldwide. There is a dire need to develop new pathogen inactivation techniques that can be used to safeguard the blood supply and to generate inactivated vaccines against these emerging viruses. Unfortunately, current techniques are inadequate because they introduce toxic/carcinogenic chemicals or alter the structure of the products, leading to loss of safety and efficacy. We have developed a novel, chemical-free ultrashort pulsed laser treatment system capable of inactivating a broad spectrum of both enveloped and non-enveloped viruses. We found that ultrashort pulsed laser treatment targets the vibrational modes of viral capsids through impulsive stimulated Raman scattering, leading to virus inactivation. Our studies reveal a capsid defect in ultrashort pulsed laser-treated viruses as a result of laser-driven aggregation of viral capsid proteins. We have applied the ultrashort pulsed laser method to pathogen reduction in
human plasma, and have demonstrated that laser treatment can inactivate medically important viruses while preserving human plasma. Furthermore, we have applied the ultrashort pulsed laser method to vaccine generation, and have shown that laser-inactivated virus vaccines have the potential to be safer and more effective than vaccines inactivated by conventional techniques. Ultrashort pulsed laser treatment represents a unique and promising new pathogen inactivation method to address the issue of emerging pathogens.
CHAPTER 1

Introduction
Summary of Doctoral Research and Dissertation

My doctoral research focuses on the discovery and development of an ultrashort pulsed laser technology for pathogen inactivation. During this doctoral study, I have explored the physical and biological mechanism of ultrashort pulsed laser inactivation of viruses. I have demonstrated the efficacy of ultrashort pulsed laser treatment in virus reduction of blood products and in the generation of safe and effective inactivated virus vaccines. The dissertation is structured as follows: Chapter 1 is an introduction to viruses, to virus inactivation methods, and to the ultrashort pulsed laser treatment method. Chapters 2-6 derive in large part from published or submitted journal manuscripts, where each chapter contains material from an article where I served as lead author. Chapter 7 presents a concise summary of the dissertation with conclusions.

Structure and Function of Viruses

Viruses are among the oldest biological entities known. Viruses are obligate parasites that rely on the replication, transcription, and translation machinery of eukaryotic or prokaryotic cells to propagate\(^1\). A virus fundamentally consists of nucleic acid (DNA or RNA) surrounded by a protein shell, termed a capsid, which serves to protect the viral genome from degradation. In a class of virus termed “non-enveloped viruses”, protein structures on the surface of capsids serve as ligands that bind to cellular receptors on host cells during the infection process. Examples of non-enveloped viruses include hepatitis A virus, parvovirus B19, adenovirus, norovirus, and poliovirus. Certain viruses belong to a different class known as “enveloped viruses”; these viruses also contain a lipid membrane, derived from a host cell, which surrounds the capsid. In
enveloped viruses, envelope proteins embedded within the lipid membrane serve as ligands for cellular binding and infection. Examples of enveloped viruses include human immunodeficiency virus (HIV), influenza virus, severe acute respiratory syndrome (SARS) virus, hepatitis C virus (HCV), West Nile virus (WNV), herpes simplex virus, and cytomegalovirus. Viruses may also contain “matrix” or “tegument” proteins associated with the capsid, which serve a variety of roles including intracellular capsid disassembly/transport. The generic structure of an enveloped virus is shown in Figure 1.

**Fig 1.** Generic structure of an enveloped virus.

At the microscopic level, viruses enter host cells by binding to receptors on the surface of cells via protein ligands on the surface of the viral capsid (in the case of non-enveloped viruses)
or on the surface of the viral envelope (in the case of enveloped viruses)\(^1\). The entry of viruses generally involves internalization into host cells by fusion or endocytosis, followed by the release of viral genomic DNA/RNA. The viral genome then utilizes endogenous cellular replication, transcription, and translation machinery to produce many copies of its genome and to express virus-encoded proteins within the cell. These viral proteins direct the assembly of new virus particles, which emerge from the cell to infect other cells. Many viruses encode their own polymerases to facilitate their replication. Many viruses also encode proteins that serve immunoevasive functions such as downregulation of immune recognition markers (MHC, NKG2D ligands etc.) to prevent the recognition and destruction of host cells by the immune system.

At the level of multicellular organisms including humans, viruses are transmitted via multiple routes including mucosal (oral, nasal, genital) and parenteral (intravenous). Oral transmission of viruses such as hepatitis A virus occurs largely through ingestion of contaminated food or water. Nasal transmission of “airborne” viruses such as influenza virus occurs via respiration of water droplets released by coughing and sneezing. Genital transmission of viruses such as herpes simplex virus occurs through sexual contact. Parenteral transmission of “blood-borne” viruses such as HIV and HCV typically occurs via intravenous drug use or blood transfusion.

Oral transmission of viruses has been largely prevented in the developed world through pasteurization techniques and water treatment/purification systems. However, transmission of both known and emerging viruses through nasal (aerosol) and genital routes is not easily preventable, and such viruses spread effectively through populations. Transmission of known
viruses through blood transfusion can be partially prevented through screening and testing of blood products, but this system cannot protect against emerging viruses or viruses existing at levels below the limits of detection of available assays.

Emerging viruses arguably pose the greatest threat to human health worldwide, and serve as a motivation for the studies in this dissertation. On the whole, the human-virus dynamic can be compared to a whac-a-mole situation in which (1) first, a new virus emerges; (2) second, steps are taken to control the spread of the infection; and (3) third, a lengthy research process ensues resulting in the production of specific antiviral medications and/or vaccines over the course of several years. In this regard, human technology is always “one step behind” viral evolution, and millions of human lives are put at risk in the interim. Therefore, a strategy to effectively address emerging viruses would be highly desirable and would have considerable impact on morbidity and mortality across the globe.

**Threat of Emerging Viruses**

Emerging viruses have been responsible for catastrophic loss of human life throughout history, and represent a global threat to human health today. For example, the 1918 influenza pandemic infected an estimated 500 million people across the world, killing nearly 5% of the world’s population\(^2\). In the 1970s, the rise of human immunodeficiency virus (HIV) and hepatitis C virus (HCV) caused millions of individuals to become infected through blood transfusions before screening and testing for these pathogens were established\(^3\). More recently, we have been confronted with the emergence of West Nile virus (WNV), severe acute respiratory syndrome
(SARS) virus, and lethal strains of influenza. These viruses can arise through biological evolution or bioterrorism, and represent a constant and enduring threat for the foreseeable future. The viruses with greatest impact on human morbidity and mortality can be divided into two groups: blood-borne viruses and airborne viruses. These groups are described separately in the following sections.

**Emerging blood-borne viruses and pathogen reduction strategies**

A diverse array of blood-borne pathogens is transmitted through transfusion of infected blood products derived from apparently healthy and asymptomatic donors. These pathogens include HIV, hepatitis B virus (HBV), HCV, parvovirus B19, dengue virus, and WNV, among many others\(^4\). The risk of transmitting known pathogens through blood transfusion has now been greatly reduced through the implementation of donor screening and nucleic acid and immunological testing. However, this system has an important limitation: new pathogens can emerge in the blood supply and pose serious threats before screening and testing methods are in place, as historically evidenced by HCV, HIV, and more recently, WNV\(^3,5\). As a dramatic example, it has been estimated that 4.8 million people acquired hepatitis through transfusions between 1970 and 1990; the majority of these cases were HCV, now the leading cause of liver transplantation in North America\(^3\). The transfusion medicine community is now challenged with a growing list of new threats, including dengue virus, chikungunya virus, malaria, babesiosis, and human herpes virus 8, to name a few\(^3\). A dilemma thus emerges. On one hand, waiting for pathogens to arise as serious threats before taking action would expose thousands or perhaps
millions of patients to harm in the interim. On the other hand, testing donors for all possible transmissible pathogens would be prohibitively expensive. These challenges attest to the need for an efficient method to inactivate known, new, or undetectable pathogens in blood products.

Pathogen reduction (PR) strategies that preemptively eliminate pathogens from blood products are an attractive solution to address the challenges associated with both known and emerging pathogens. However, the currently licensed PR methods, including solvent-detergent (SD) and ultraviolet (UV)-activated photochemicals, have seen limited use in the United States due in part to concerns of adverse effects associated with chemicals used during these PR processes. In addition, these methods are ineffective against non-lipid-enveloped viruses such as hepatitis A virus. Furthermore, with these methods, the introduction and removal of chemicals are additional steps that increase the cost of their implementation. In these regards, a chemical-free PR technology that broadly inactivates pathogens would be advantageous. To this end, UV radiation alone has been tested, but was found to be limited by pathogen resistance and unacceptable damage to blood components. Therefore, it is important to develop new PR technologies that can circumvent these limitations and provide long-term safety to the United States blood supply. The ideal PR technology would be a method capable of inactivating a broad spectrum of pathogens while retaining the function of blood products, and without the need to introduce biological or chemical agents. Figure 2 illustrates the concept of pathogen reduction of blood products.
Fig 2. The concept of pathogen reduction of blood products.

Emerging airborne viruses and vaccine generation strategies

Viruses that can be transmitted through the air pose a high pandemic risk in the modern era of international travel. Important examples include SARS virus, H1N1 swine influenza, H5N1 avian influenza, and more recently, a novel Middle East Respiratory Syndrome (MERS) virus. An emerging virus can quickly spread from its source to threaten the global population in a matter of weeks, as was observed for the SARS outbreak in 2003. On one hand, quarantine measures and temperature scanners in airports provide some protection against spread, but the pandemic threat remains with enormous medical, social, and economic consequences. On the other hand, the design of specific antiviral drugs is a lengthy process, is prone to the inevitable rise of resistance, and cannot address the initial stages of an outbreak.

In a scientific context, the most logical and cost-effective method to protect against a viral epidemic is to rapidly develop a vaccine to that virus. A variety of strategies including whole inactivated virus (WIV), virus-like particles, and viral protein subunits have been used for vaccine production. Of these, WIV vaccines have been used successfully since the mid-20th
century to control important diseases such as influenza and polio. WIV vaccines consist of whole virus particles that have been killed (rendered non-infectious) by chemical or physical methods, and they have several key advantages over other vaccine strategies. First, WIV vaccines contain the entire repertoire of virion-associated antigens and immunostimulatory elements, and thus can elicit stronger and broader immune responses in naïve subjects compared to split virus (SV) or subunit (SU) vaccines\textsuperscript{8-13}, which are detergent-/ether-disrupted virus or purified viral proteins, respectively. The superior immunogenicity of WIV vaccines has been attributed to the presence of viral nucleic acids that trigger innate immune activation via pattern recognition receptors\textsuperscript{14}. Second, WIV vaccines are capable of inducing T helper type 1 (Th1)-skewed immune responses\textsuperscript{9,10}, which are favorable for the effective containment of persistent intracellular pathogens and for cross-protection against rapidly mutating pathogens. Importantly, WIV vaccines are straightforward to design since no specific foreknowledge of antigens is required. These properties make WIV vaccines an attractive general strategy to rapidly produce vaccines against known and emerging pathogens.

Numerous pathogen inactivation methods have been explored for the development of WIV vaccines. The most established method is formalin inactivation\textsuperscript{15}. However, despite its widespread use, it is well known that formalin causes extensive molecular damage via crosslinking of proteins, thus destroying the structure of antigens that are important for inducing an appropriate antibody response\textsuperscript{16,17}. Formalin treatment also generates carbonyl groups on proteins, which may be responsible for hypersensitivity and disease worsening associated with formalin-inactivated respiratory syncytial virus (RSV) and measles vaccines\textsuperscript{18,19}. Furthermore, the formalin method introduces a carcinogen into the vaccine which cannot be completely
removed; this poses a potential risk for adverse effects in humans. The chemical removal process introduces an extra step in manufacturing that inevitably leads to loss in product. Other chemicals have been explored for viral inactivation, including β-propiolactone and hydrogen peroxide\textsuperscript{20}. However, as with formalin, chemical or free radical-induced modification of viral proteins has similar limitations to formalin treatment.

Chemical-free pathogen inactivation methods could potentially minimize some of these concerns. To this end, several physical methods including heat\textsuperscript{21,22}, ultraviolet (UV) light\textsuperscript{23,24}, and gamma irradiation\textsuperscript{25-28} have been investigated for WIV vaccine generation. However, these techniques are also limited because they damage virions through the alteration of antigenic structures, reducing the efficacy of the vaccine. Heat causes the denaturation of protein antigens by disrupting hydrogen bonds and non-covalent interactions. UV irradiation, which inactivates pathogens by DNA damage through dimerization of adjacent pyrimidines\textsuperscript{29} and through generation of reactive oxygen species\textsuperscript{30}, is strongly absorbed by the aromatic side chains within proteins leading to disruption of secondary or tertiary protein structures. In addition, the efficacy of UV light is limited, as there is already evidence of resistance to UV treatment among blood borne viruses such as HIV\textsuperscript{31}. Finally, although gamma irradiated vaccines have shown cross-protection and efficacy in mice\textsuperscript{26,28}, the high-energy ionizing gamma rays cause indiscriminate breakage of covalent and ionic bonds, leading to extensive molecular damage to proteins and potentially even the generation of new structures with unknown effects in humans\textsuperscript{32,33}. In these regards, the ideal technology for generating WIV vaccines should be able to inactivate the virus without compromising viral immunostimulatory structures, and without the need to introduce
biological or chemical agents. Figure 3 illustrates the concept of virus inactivation for vaccine generation.

![Diagram showing the process of inactivating infectious virus to killed virus through treatment, leading to effective vaccines and vaccination.]

**Fig 3.** The concept of virus inactivation for vaccine generation.

**A novel ultrashort pulsed laser method for pathogen inactivation**

As described previously, the existing techniques for pathogen inactivation including chemical methods (SD, amotosalen, formalin, β-propiolactone, etc.) and physical methods (UV, heat, gamma radiation) are all limited by unacceptable concerns of side effects and excess collateral damage to blood products and vaccines. The ideal pathogen inactivation technology would be a one-step process that inactivates a broad spectrum of viruses by targeting a universal feature of viruses, without the need to introduce biological or chemical agents.

In this regard, ultrashort pulsed lasers operating in the visible/near-infrared wavelength region are a potentially ideal pathogen inactivation method. We have recently discovered
through empirical observation that ultrashort pulsed laser treatment inactivates a wide variety of viruses including enveloped, non-enveloped, DNA, and RNA viruses\textsuperscript{34-43} (Table 1). Visible ultrashort pulsed laser treatment has several theoretical features that make it attractive strategy for pathogen inactivation. First, it targets a universal feature of viruses, the capsid, by excitation of capsid-specific vibrational modes through impulsive stimulated Raman scattering. The vibrational frequency of viral capsids is a fundamental physical property that is unlikely to change through mutations in viral nucleic acid; thus the chance of viral escape/resistance is minimal. Second, the ultrashort pulsed laser lacks the energy to disrupt covalent bonds in biological systems, resulting in minimal collateral damage to the treated product. Third, it does not involve introducing any toxic or carcinogenic chemicals. Fourth, it is environmentally friendly and does not involve the use of mercury that is used in UV lamps, for instance.

*Table 1. Killing efficacy for a variety of viruses using a 425 nm femtosecond pulsed laser.*

<table>
<thead>
<tr>
<th>Microorganism</th>
<th>Properties</th>
<th>Load Reduction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human Immunodeficiency Virus (HIV)</td>
<td>Enveloped, ssRNA</td>
<td>$10^4$</td>
</tr>
<tr>
<td>Influenza Virus</td>
<td>Enveloped, ssRNA</td>
<td>$10^5$</td>
</tr>
<tr>
<td>Encephalomyocarditis virus (EMCV)</td>
<td>Non-enveloped, ssRNA</td>
<td>$10^4$</td>
</tr>
<tr>
<td>Murine norovirus (MNV)</td>
<td>Non-enveloped, ssRNA</td>
<td>$10^3$</td>
</tr>
<tr>
<td>Hepatitis A virus (HAV)</td>
<td>Non-enveloped, ssRNA</td>
<td>$10^3$</td>
</tr>
<tr>
<td>Human Papillomavirus (HPV)</td>
<td>Non-enveloped, dsDNA</td>
<td>$10^5$</td>
</tr>
<tr>
<td>M13 bacteriophage</td>
<td>Non-enveloped, ssDNA</td>
<td>$10^5$</td>
</tr>
</tbody>
</table>
Figure 4a provides a general schematic diagram of a laser. At the fundamental level, a laser (“light amplification by stimulated emission of radiation”) consists of a gain medium (solid, liquid, or gas) within a chamber, which is excited by an energy source. As electrons within the gain medium fall back to ground state they emit energy in the form of photons. These photons are directed between two mirrors, one totally reflecting and the other partially reflecting to allow a fraction of the beam to exit the chamber. A pulsed laser beam can be generated by the incorporation of a mode-locker such as a standing acoustic wave, or by self mode-locking via the use of a Kerr medium as in the case of the Titanium Sapphire (Ti-Sapphire) laser.
Figure 4b illustrates the ultrashort pulsed laser treatment system we have used for our studies. We employed a diode-pumped cw mode-locked Ti-sapphire laser as the excitation source. The laser produces a continuous train of 60 fs pulses at a repetition rate of 80 MHz. The output of the second harmonic generation (SHG) system of the Ti-sapphire laser is used to irradiate the sample. The excitation laser is chosen to operate at a wavelength of $\lambda = 425$ nm and with an average power of approximately 150 mW. It has a pulse width of full-width at half maximum (FWHM) = 100 fs. An achromatic lens is used to focus the laser beam into a spot about 100 $\mu$m in diameter within the sample volume. Samples of virus suspended in phosphate buffered saline (PBS) or human plasma are irradiated in glass vials. In order to facilitate the interaction of laser with the virus, a magnetic stirring system is used so that the virus enters the laser-focused volume and interacts with the photons. Controls (untreated virus samples) are similarly stirred. After laser treatment, infectivity assays, gel electrophoretic analysis, imaging,
etc. are performed on control (untreated) or laser-treated virus samples, or on cells infected with control or laser-treated virus.

In this dissertation, I explore the physical and biological mechanisms of inactivation to confirm that the ultrashort pulsed laser treatment inactivates viruses through impulsive stimulated Raman scattering. Furthermore, I demonstrate application of ultrashort pulsed laser treatment in pathogen reduction of human blood products and generation of inactivated vaccines. This novel technology represents a potentially effective strategy to protect the human world against emerging viruses.

References


Murphy, B. R. & Walsh, E. E. Formalin-inactivated respiratory syncytial virus vaccine induces antibodies to the fusion glycoprotein that are deficient in fusion-inhibiting activity. *Journal of Clinical Microbiology* 26, 1595-1597 (1988).


Tseng, K. T. et al. Studies of inactivation of encephalomyocarditis virus, M13 bacteriophage, and Salmonella typhimurium by using a visible femtosecond laser: insight


CHAPTER 2

Inactivation of Enveloped Virus by Laser-Driven Protein Aggregation
Chapter 2 is a reformatted version of a published manuscript: “Shaw-Wei D. Tsen, Travis Chapa, Wandy Beatty, et al. Inactivation of enveloped virus by laser-driven protein aggregation. Journal of Biomedical Optics 17(12), 128002 (2012).” Under Dr. Samuel Achilefu’s supervision, my contributions to this work included designing and developing the ultrashort pulsed laser technology, carrying out the characterization and laser inactivation experiments, interpreting and analyzing data, and writing the manuscript. In this Chapter, I discuss my work in the characterization of the molecular mechanism of virus inactivation using a visible ultrashort pulsed laser.
Abstract

Ultrafast lasers in the visible and near-infrared range have emerged as a potential new method for pathogen reduction of blood products and pharmaceuticals. However, the mechanism of enveloped virus inactivation by this method is unknown. Here, we report the inactivation as well as the molecular and structural effects caused by visible (425 nm) femtosecond laser irradiation on murine cytomegalovirus (MCMV), an enveloped, double-stranded DNA virus. Our results show that laser irradiation (1) caused a 5-log reduction in MCMV titer; (2) did not cause significant changes to the global structure of MCMV virions including membrane and capsid, as assessed by electron microscopy; (3) produced no evidence of double-strand breaks or crosslinking in MCMV genomic DNA; and (4) caused selective aggregation of viral capsid and tegument proteins. We propose a model in which USP laser irradiation induces partial unfolding of viral proteins by disrupting hydrogen bonds and/or hydrophobic interactions, leading to aggregation of closely associated viral proteins and inactivation of the virus. These results provide new insight into the inactivation of enveloped viruses by visible femtosecond lasers at the molecular level, and help pave the way for the development of a new ultrafast laser technology for pathogen reduction.


**Introduction**

Pathogen reduction (PR), which aims to proactively eliminate infectious agents from blood products, is an attractive strategy to address the threat of known and emerging pathogens and ensure the continued safety of the blood supply. However, the various PR methods explored to date suffer from limitations that prevent their widespread use and acceptance by the transfusion medicine community. Clinically tested PR techniques for human plasma include solvent-detergent (SD) treatment\(^1\), visible light-activated sensitizers such as methylene blue\(^2\), and ultraviolet (UV) light-activated photochemicals such as riboflavin and amotosalen\(^3\)\(^{-}\)\(^{11}\). All current techniques involve the introduction of chemicals with risks of unknown or unpredictable side effects. These side effects include immune reactions, carcinogenicity, or loss of coagulation factors in the product, all of which can lead to adverse consequences in patients\(^12\). SD treatment is also limited because it cannot inactivate non-enveloped viruses, and thus is ineffective against many transfusion-transmitted pathogens such as parvovirus B19 and hepatitis A virus (HAV)\(^12\). Furthermore, with all of the abovementioned methods, the introduction and subsequent removal of chemicals is an additional step that adds to the cost of implementing the PR technology.

Short wavelength UV (UVC) radiation has been tested as an alternative, chemical-free PR technology. UVC radiation inactivates pathogens by DNA damage through dimerization of adjacent pyrimidines\(^13\) as well as generation of reactive oxygen species\(^14\). UVC treatment has shown effects against certain viruses and bacteria\(^15\)\(^{-}\)\(^{17}\). However, there is already evidence of resistance to UVC among blood borne pathogens such as HIV\(^17\). Moreover, UVC is strongly absorbed by proteins and has been shown to damage plasma components\(^18\) and cause platelet
aggregation\textsuperscript{19}. Thus, there is a need to develop a new chemical-free PR technique with broader pathogen coverage and minimal effects on the blood product.

In this regard, ultrafast lasers in the visible and near-infrared range are a potentially ideal approach for PR. Visible/near-infrared ultrafast laser irradiation does not cause ionization effects, which can damage the blood product. It does not introduce potentially toxic or carcinogenic chemicals, and thereby has minimal concern of adverse effects. Our group has recently shown femtosecond laser irradiation to be effective in inactivating (achieving 3-5 log reduction of) a broad spectrum of viruses\textsuperscript{20} including human immunodeficiency virus (HIV)\textsuperscript{21,22}, human papillomavirus (HPV)\textsuperscript{22}, encephalomyocarditis virus (EMCV)\textsuperscript{23}, M13 bacteriophage\textsuperscript{23-27}, and tobacco mosaic virus (TMV)\textsuperscript{22}. More importantly, femtosecond laser irradiation at sufficient power to kill the abovementioned viruses does not kill human cells\textsuperscript{27} and does not appear to damage either bovine serum albumin (BSA) protein or single stranded DNA\textsuperscript{23}.

Human cytomegalovirus (HCMV) is a widespread pathogen responsible for multiple important diseases. It is the leading viral cause of congenital diseases in newborns, a common cause of opportunistic infections in AIDS and transplant patients, and a potential risk factor in certain cardiovascular diseases\textsuperscript{28,29}. Significant limitations are seen with current antiviral therapeutics\textsuperscript{30-33}, and there are considerable needs for new treatments of HCMV disease\textsuperscript{34}. The severity of medical problems associated with HCMV in these vulnerable populations underlies the necessity for ensuring that blood products are safer for this group of patients. Murine cytomegalovirus (MCMV) is now widely used as a surrogate model for HCMV because of its robust replication, its tractable genetic systems, the availability of many reagents for both the
virus and host, and access to an animal model for in vivo experimentation which is impossible for HCMV.

We note that the specific effects of femtosecond laser irradiation on viral membranes, capsids, and nucleic acids at the molecular level remain unclear. This information is essential for the optimization, application, and approval of femtosecond lasers for use in PR of therapeutics including pharmaceuticals and blood products. Toward these goals, we report the inactivation of MCMV, a model herpesvirus, by using a 425 nm-femtosecond laser and determine the molecular effects of laser irradiation on MCMV virions, viral genomic DNA, and virion-associated proteins. In remarkable contrast to the atomic force microscope (AFM) images of non-enveloped viruses such as M13 bacteriophage and TMV showing that the capsids were broken by the USP laser irradiation presented in the previous work, our TEM images revealed that the USP laser did not break/dissociate the capsid of MCMV. We propose a novel mechanism for the enveloped virus inactivated by visible femtosecond lasers through induction of viral protein aggregation. By correlating viral inactivation with the observed structural and molecular effects, a better insight into the inactivation of enveloped viruses by femtosecond laser irradiation was obtained.

**Materials and Methods**

**Cells and viruses.** Murine embryonic fibroblast 10.1 (MEF 10.1) cells were cultured in Dulbecco’s Modified Eagle Medium (DMEM), supplemented with 10% fetal calf serum, 1mM
sodium pyruvate, and nonessential amino acids. GFP-expressing MCMV virus (hereafter referred to as “MCMV”) was generated as previously described\textsuperscript{35}. To produce viral stocks, MEF 10.1 cells were infected with MCMV at a low multiplicity of infection. Cell supernatants were harvested 24 hours post-infection after 100% cytopathic effect and cleared of cell debris by centrifugation. Extracellular virions were pelleted by ultracentrifugation with sorbitol cushion and resuspended in phosphate-buffered saline (PBS). Viral titers were determined in quadruplicate using a median tissue culture infectious dose (TCID\textsubscript{50}) assay.

**Femtosecond laser irradiation.** The experimental setup for laser irradiation is shown in Figure 1. The excitation source employed in this work was a diode-pumped cw mode-locked Ti-sapphire laser. The laser produced a continuous train of 60 fs pulses at a repetition rate of 80 MHz. The output of the second harmonic generation (SHG) system of the Ti-sapphire laser was used to irradiate the sample. The excitation laser was chosen to operate at a wavelength of $\lambda = 425$ nm and with an average power of approximately 150 mW. It has a pulse width of full-width at half maximum (FWHM) = 100 fs. An achromatic lens was used to focus the laser beam into a spot about 100 $\mu$m in diameter within the sample volume. Samples of virus suspended in phosphate buffered saline (PBS) at a concentration of $\sim 2 \times 10^7$ pfu/ml were irradiated for 1.5 hours. In order to facilitate the interaction of laser with the virus, a magnetic stirring system was used so that the virus would enter the laser-focused volume as described above and interact with the photons. Controls were similarly stirred. Irradiation was carried out at 22°C and with the single laser beam excitation. After laser irradiation, samples were immediately stored at -80°C.
**Fig 1.** Experimental setup for femtosecond laser treatment. An 850 nm (near-infrared) laser beam was frequency doubled to produce 425 nm (visible) irradiation. Samples were magnetically stirred to expose the virus to the laser-focused volume.

**TCID\textsubscript{50} assays.** TCID\textsubscript{50} assays were performed to determine reduction in viral titers following laser irradiation. MEF 10.1 cells were seeded into 96 well plates at a density of \(1.25 \times 10^5\) cells/ml and incubated overnight. Cells were approximately 80\% confluent at the time of infection. Laser-treated or control (untreated) virus were serially diluted and added to cells, and cells were incubated for 4 days. Viral titers were determined on day 4 post-infection by scoring each well for GFP-positive cells using a fluorescent microscope.

**Electron microscopy.** Laser-treated or control (untreated) MCMV virions were allowed to absorb onto formvar/carbon-coated copper grids for 10 min. Grids were washed in distilled water and stained with 1\% phosphotungstic acid (Electron Microscopy Sciences, Hatfield, PA) for 1 min. Excess liquid was gently wicked off and grids were allowed to air dry. Samples were viewed on a JEOL 1200EX transmission electron microscope (JEOL USA, Peabody, MA) at an accelerating voltage of 100kV. Images were acquired with a XR80M-B 8 megapixel CCD camera system (Advanced Microscopy Techniques Corporation, Woburn, MA).
**Purification of MCMV DNA.** Laser-treated or control (untreated) MCMV virions were treated with DNase I for 30 minutes at 37°C, and then transferred to 75°C to inactivate DNase I. Viral membranes were lysed using a lysis buffer (800 mM NaCl, 20 mM Tris [pH 8.0], 20 mM EDTA, 0.4% sodium dodecyl sulfate (SDS)), and viral capsids were digested by incubation with proteinase K (0.2 mg/ml) at 55°C overnight. DNA was extracted with phenol-chloroform, precipitated with isopropanol, and centrifuged at 13,000×g for 30 minutes at 4°C. The pellets were washed with 70% ethanol and resuspended in water. For subsequent agarose gel electrophoresis, MCMV DNA samples were left intact or digested using EcoRI or HindIII restriction enzymes for 5 hours at 37°C prior to gel loading.

**Agarose gel electrophoresis.** Agarose gels, 0.6% and 5 mm in thickness, were cast in a full-length gel apparatus. The MCMV DNA samples were mixed with loading buffer and then loaded into wells. Electrophoresis was carried out at 30 V/cm overnight. The gel was then stained with ethidium bromide for 30 minutes, destained for 3 hours and visualized under UV illumination.

**Protein gel electrophoresis.** Protein concentration of viral solutions was determined by Bradford assay (colorimetric protein assay kit, Bio-Rad). Solutions of laser-treated or control (untreated) virus containing equivalent quantities of protein were boiled in reducing loading buffer and separated on a 10% SDS-PAGE gel. Protein bands were visualized with Coomassie blue staining (LabSafe Gel Blue, G-Biosciences).
Mass spectrometry analysis. Gel slices were excised manually and submitted to Midwest Bio Services, LLC (Overland Park, KS) for trypsin digest followed by nano LC-MS/MS analysis and protein identification. For details on the protocol, please refer to http://www.midwestbioservices.com/proteinid.html.

Dynamic Light Scattering. mAb(04) samples in buffer solution (50 mM sodium acetate, PH 7.0) were from Enzo life Sciences (Farmingdale, NY). BSA samples in buffer solution (50 mM sodium acetate, PH 7.0) were from Thermal Scientific Inc. (Mansfield, TX). The dynamic light scattering (DLS) experiments were carried out by using a 90Plus Particle Size Analyzer from Brookhaven Instruments Corp. (Holtsville, NY).

Statistics. Differences between mean TCID$_{50}$ titers of control and laser-treated virus were analyzed by Student’s t-test. p<0.05 was used as a threshold for statistical significance.
Results

MCMV is efficiently inactivated by femtosecond laser irradiation

We have previously shown that a variety of enveloped/non-enveloped, single-stranded DNA/RNA viruses can be inactivated by femtosecond laser irradiation\textsuperscript{21-27}. Therefore, we sought to demonstrate that irradiation with a femtosecond laser at a similar laser power (150 mW) could also inactivate MCMV, an enveloped, double-stranded DNA virus. The laser setup is illustrated in Figure 1. For all experiments in this report, we used a previously established GFP-expressing MCMV\textsuperscript{35} for ease of detection of infectious virus by TCID\textsubscript{50} assay. As shown in Figure 2, irradiation with a 425 nm-femtosecond laser at 150 mW caused a 5-log reduction in MCMV titer relative to the control (non-irradiated) MCMV (p=0.0072). The titers of all laser-treated samples were at or near the limit of detection of the assay. This is consistent with the inactivation efficiency (3-5 log) for other viruses using the same laser conditions. Therefore, we conclude that 425 nm-femtosecond laser irradiation is an effective method to inactivate MCMV.
Fig 2. Inactivation of MCMV using a femtosecond laser. Femtosecond laser-induced reduction in viral titer was assessed by TCID$_{50}$ assay. Error bars indicate SEM.

MCMV global virion structure is preserved after femtosecond laser irradiation

We used negative-stain transmission electron microscopy (TEM) to investigate whether the envelope or capsid structures of MCMV virions were affected by 425 nm-femtosecond laser irradiation at an average laser power of 150 mW. As shown in Figure 3, no clear differences in the global appearance of the envelope or capsid structure of virions in control (non-irradiated) relative to laser-irradiated groups were observed. At this resolution, we could not find any evidence of MCMV capsid disintegration after laser irradiation. These experimental results suggest that the global envelope and capsid structures of MCMV remained intact after 425 nm-femtosecond laser irradiation.
**Fig 3.** Preservation of MCMV global virion structure after femtosecond laser treatment. Representative electron microscopy images are shown of control and laser-treated virions (150 to 200 nm in diameter) at both 50,000x and 150,000x magnification, showing no clear differences in the global appearance of viral enveloped and capsid structures after treatment.

**MCMV genomic DNA structure is preserved after femtosecond laser irradiation**

We used gel electrophoresis to assess whether MCMV genomic double-stranded DNA was covalently damaged by 425 nm-femtosecond laser irradiation at an average power of 150 mW. DNA was electrophoresed intact or after digestion with restriction enzymes. If double strand breakage or extensive crosslinking occurred, we would expect to see a change in the banding pattern of laser-irradiated DNA. As shown in Figure 4, viral genomic DNA from
femtosecond laser-irradiated MCMV exhibited identical banding patterns to genomic DNA from control (non-irradiated) MCMV. These experimental findings are consistent with our previous reports that 425 nm-femtosecond laser irradiation does not cause strand breaks in the single-stranded DNA of M13 bacteriophage. These data indicate that 425 nm-femtosecond laser irradiation did not cause double strand breaks or crosslinking of the MCMV genome.

Fig 4. Preservation of MCMV genomic DNA integrity after femtosecond laser treatment. Agarose gel analysis was performed on genomic DNA isolated from control or irradiated MCMV, showing essentially identical banding patterns, which suggests a lack of double-strand breaks or crosslinking of viral DNA after femtosecond laser treatment. DNA was electrophoresed intact or after digestion using restriction enzymes. For clarity, image contrast and sharpness were enhanced uniformly across the entire image using Photoshop.
Femtosecond laser irradiation causes selective aggregation of MCMV capsid and tegument proteins

We employed SDS-PAGE to determine the effects of femtosecond laser irradiation on MCMV virion-associated proteins. Interestingly, several protein bands from laser-irradiated virions showed substantially reduced intensities relative to those of control (non-irradiated) virions (Figure 5). Since our 425-nm laser treatment lacks the energy required to disrupt the covalent bonds commonly found in proteins, fragmentation of viral proteins or the production of covalent cross-linkages were unlikely scenarios.

**Fig 5.** Aggregation of MCMV virion proteins after femtosecond laser treatment. SDS-PAGE analysis was performed on control and laser-treated virions. The arrow denotes a high molecular weight aggregate formed by proteins in the laser-treated group. The aggregate was excised for mass spectrometry analysis. For clarity, image contrast and sharpness were enhanced uniformly across the entire image using Photoshop.
As shown by the arrow in Figure 5, a high molecular weight protein aggregate was consistently detected in the laser-irradiated group. Based on this observation, we reasoned that laser irradiation may have caused aggregation of viral proteins. Therefore, the aggregate was excised from the gel and submitted for protein identification. The list of MCMV proteins identified is shown in Table 1, and the quantitative mass spectrometry data can be found in Supplementary Table 1 (http://www.ncbi.nlm.nih.gov/pmc/articles/PMC3518210/). The aggregate contained predominantly MCMV virion-associated capsid and tegument proteins. The identified MCMV proteins have previously been detected in MCMV virions\textsuperscript{36}. In contrast, the MCMV envelope glycoproteins such as glycoprotein B\textsuperscript{37}, glycoprotein H\textsuperscript{38} and glycoprotein M\textsuperscript{39} were not detected in the aggregate. These data indicate that femtosecond laser irradiation causes selective aggregation of MCMV capsid and tegument proteins, an effect that may hinder viral capsid function (i.e. uncoating) and contribute to inactivation of the virus.

\textbf{Table 1.} MCMV proteins from the aggregate identified by LC-MS/MS.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Accession</th>
<th>Comment</th>
<th>HCMV homologue</th>
<th>p-value</th>
<th>No. of peptides</th>
</tr>
</thead>
<tbody>
<tr>
<td>M25</td>
<td>gi:190886806</td>
<td>Tegument protein\textsuperscript{40}</td>
<td>UL25</td>
<td>4.42×10\textsuperscript{-6}</td>
<td>29</td>
</tr>
<tr>
<td>M32</td>
<td>gi:190886815</td>
<td>(HCMV: pp150)\textsuperscript{41}</td>
<td>UL32</td>
<td>6.17×10\textsuperscript{-8}</td>
<td>18</td>
</tr>
</tbody>
</table>
### Discussion

In this report we have examined the effects of femtosecond lasers on MCMV structures at the molecular level. The capability of 425 nm-femtosecond laser treatment to inactivate a spectrum of different viruses and bacteria has been well documented\(^\text{20-27}\). In these previous studies, it was demonstrated that the non-enveloped viruses such as M13 bacteriophages were

<table>
<thead>
<tr>
<th>M35</th>
<th>gi:190886819</th>
<th>UL25 family member, virulence factor(^\text{42})</th>
<th>UL35</th>
<th>3.54×10(^{-6})</th>
<th>4</th>
</tr>
</thead>
<tbody>
<tr>
<td>M44</td>
<td>gi:90954727</td>
<td>DNA binding phosphoprotein(^\text{43})</td>
<td>UL44</td>
<td>1.57×10(^{-5})</td>
<td>4</td>
</tr>
<tr>
<td>M80</td>
<td>gi:157676178</td>
<td>Assembly protein-protease(^\text{44})</td>
<td>UL80</td>
<td>4.82×10(^{-4})</td>
<td>4</td>
</tr>
<tr>
<td>M82</td>
<td>gi:157676179</td>
<td>Upper matrix phosphoprotein, pp71(^\text{45})</td>
<td>UL82</td>
<td>3.63×10(^{-4})</td>
<td>2</td>
</tr>
<tr>
<td>M83</td>
<td>gi:1532178</td>
<td>Lower matrix phosphoprotein, pp65(^\text{45})</td>
<td>UL83</td>
<td>8.19×10(^{-4})</td>
<td>2</td>
</tr>
<tr>
<td>M86</td>
<td>gi:157676184</td>
<td>(HCMV: major capsid protein)(^\text{46})</td>
<td>UL86</td>
<td>9.09×10(^{-7})</td>
<td>43</td>
</tr>
<tr>
<td>M94</td>
<td>gi:157676191</td>
<td>(HCMV: virion-associated protein)(^\text{47})</td>
<td>UL94</td>
<td>1.02×10(^{-5})</td>
<td>6</td>
</tr>
</tbody>
</table>
inactivated by the USP laser irradiation through breaking/dissociation of their capsids. However, the inactivation mechanism for the enveloped viruses remains unexplored. In this work, we demonstrate that in great contrast to the non-enveloped virus such as M13, whose capsid was broken/dissociated by the USP laser irradiation, our TEM images suggest that the USP laser does not break/dissociate the capsid of the enveloped virus like MCMV. This indicates that the inactivation mechanism for non-enveloped virus is drastically different from that of an enveloped virus. We attribute the inactivation mechanism for MCMV by the USP laser to the aggregation of proteins within the virion.

Interestingly, our experimental results suggest that laser irradiation causes selective aggregation of viral capsid and tegument proteins. It has been suggested that partial unfolding of proteins is required for protein aggregation.\textsuperscript{48} We posit that femtosecond laser irradiation disrupts hydrogen bonds and/or hydrophobic interactions in viral proteins through the impulsive stimulated Raman scattering process.\textsuperscript{22,23} Although the reformation time for broken hydrogen bonds/hydrophobic contacts is believed to be short (of the order of 10 picoseconds),\textsuperscript{49,50} there is a significant chance for the MCMV capsid and tegument proteins to become aggregated since they are confined within a small volume in the virion. This explains the observation of aggregated proteins under femtosecond laser irradiation. As a result, laser-induced aggregation of MCMV capsid and tegument proteins may hinder the function of the viral capsid (i.e. uncoating) and contribute to virus inactivation. The concentration dependence of protein aggregation may thus provide a window for selectively damaging virions while leaving mammalian proteins intact.\textsuperscript{23}
To get better insight into the proposed protein aggregation model for enveloped virus inactivation, we have also performed DLS experiments for a variety of proteins in their buffer solutions: (i) on the concentration dependence of aggregation for monoclonal antibody 04; (ii) on the aggregation of BSA proteins; and (iii) on the aggregation of the mixture of BSA and mAb04, under the same experimental conditions as MCMV. The integrated area under the primary peak – the monomer, which happens at about 12 nm, 6 nm in diameter for mAb04 and BSA, respectively, divided by the total integrated area is taken as the percentage of the non-aggregated protein. The integrated area under the higher size/mass region divided by the total area is considered as the aggregated percentage.

Table 2. Dynamic Light Scattering data for a variety of laser-treated proteins in buffered solution.
The results, summarized in Table 2, indicate that (1) the structure of non-aggregated protein is not compromised; (2) the protein aggregation effect by irradiation of the USP laser depends on the type of proteins; apparently, BSA is much more stable than mAb04 and exhibits very little aggregation upon USP laser irradiation; (3) the aggregation effect depends on protein concentration, with higher concentration tending to aggregate more; (4) mixing the stable protein (BSA) with a less stable one (mAb04) does not help stabilize the less stable protein. This information further supports our proposed model that the USP laser first unfolds the proteins by disrupting their hydrogen bonds/hydrophobic contacts. The unfolded proteins then aggregate before the rapid reformation of these weak bonds. The higher protein concentration means they are closer to each other and as a result have a higher chance of aggregating. We note that under our experimental conditions, the proteins were completely dissolved in their buffer solutions, as evidenced by the almost 99% of monomer (non-aggregated proteins) in the solutions. In other words, our interpretations of protein aggregation by USP laser irradiation were not affected by the problem of solubility of the proteins in the buffer solution. In addition, the two proteins -- BSA and mAb04 -- were chosen for the DLS experiments because neither of them absorb near 425 nm. As a matter of fact, we measured the temperature of the solutions during the laser irradiation experiments with a thermal couple immersed into the solution. The temperature of the solution rose no more than 2 degrees centigrade. Since the denature temperature of both proteins is around 60 centigrade, our results cannot be due to the heating effects.

We notice that in contrast to the atomic force microscope images of non-enveloped viruses such as M13 bacteriophage and TMV showing that the capsids were broken by the USP laser irradiation presented in the previous work, our TEM images indicate that the USP laser
does not break/dissociate the capsid of the enveloped virus like MCMV. Based upon our results from DLS experiments, we propose, for enveloped viruses such as MCMV, that the USP laser partially unfolds both the protein unit of which the capsid is made up without dissociating the capsid and the tegument proteins by breaking some of their hydrogen bonds/hydrophobic contacts. These proteins then form aggregates before the reformation of the weak non-covalent bonds.

In this study, the treated volume is about 100 $\mu l$. To scale up our approach for the disinfection of blood products, we suggest the use of a syringe pump-capillary configuration in which the treated solution is forced through a capillary of the order of 1mm in inner diameter. The laser beam will be adjusted to have the same diameter and passes through the capillary in a perpendicular geometry. A much more powerful commercially available USP laser system with an average power of the order of 10W can be used for laser irradiation.

The USP laser technology presented here can be readily used for the disinfection of pharmaceuticals, which typically do not contain hemoglobin. The application of this technology to the disinfection of blood products can be done with USP lasers operating at a wavelength of about 700 nm where the absorption of hemoglobin is a minimum and the potential damaging effects can be minimized. In conclusion, we report the first experimental evidence of inactivation of an enveloped virus – MCMV by the USP laser. The molecular and structural effects caused by 425 nm-femtosecond laser irradiation on MCMV are presented and analyzed. In contrast to the atomic force microscope images of non-enveloped viruses such as M13 bacteriophage and TMV showing that the capsids were broken by the USP laser irradiation presented in the previous
work, our TEM images revealed that the USP laser did not break/dissociate the capsid of MCMV. A novel mechanism for the inactivation of an enveloped virus by visible femtosecond lasers through induction of viral protein aggregation was proposed. By correlating viral inactivation with the observed structural and molecular effects, a better insight into the inactivation of enveloped viruses by femtosecond laser irradiation was obtained. Furthermore, continued exploration of this laser pathogen inactivation technology is expected to generate applications including sterilization of pharmaceuticals, blood products, and medical equipment.

Acknowledgments

We would like to thank Irina Sorokina (Midwest Bio Services, LLC, Overland Park, KS) for mass spectrometry analysis. This work was supported in part by the Mallinckrodt Institute of Radiology Development Fund, NIH grant R33 CA123537, NHLBI Ruth L. Kirschstein NRSA F30 grant HL116183-01 (Shaw-Wei Tsen), and Public Health Service grant R01CA120768 (Dong Yu).

References


CHAPTER 3

Studies of the Inactivation Mechanism of Non-Enveloped Icosahedral Viruses by a Visible Ultrashort Pulsed Laser
Chapter 3 is a reformatted version of a published manuscript: “Shaw-Wei D. Tsen, David H. Kingsley, Christian Poweleit, et al. Studies of inactivation mechanism of non-enveloped icosahedral viruses by a visible ultrashort pulsed laser. Virology Journal 11:20 (2014).” Under Dr. Samuel Achilefu’s supervision, my contributions to this work included designing and developing the ultrashort pulsed laser technology, carrying out the laser inactivation experiments, interpreting and analyzing data, and writing the manuscript. In this Chapter, I discuss my work in the characterization of the physical mechanism of virus inactivation using a visible ultrashort pulsed laser.
Abstract

Potential contamination of pharmaceuticals, biologicals, and uncooked foods with viruses is a critical problem. Low-power ultrashort pulsed (USP) lasers operating at wavelengths of 425 nm and in the near-infrared region have been shown to effectively inactivate viruses such as human immunodeficiency virus (HIV), M13 bacteriophage, and murine cytomegalovirus (MCMV). It was shown previously that non-enveloped, helical viruses such as M13 bacteriophage were inactivated by a USP laser through an impulsive stimulated Raman scattering (ISRS) process. However, the inactivation mechanism for a clinically important class of viruses – non-enveloped, icosahedral viruses – remains unknown. In this paper, we report examination of several possible inactivation mechanisms for murine norovirus-1 (MNV-1), a non-enveloped, icosahedral virus, and present evidence that supports ISRS as the most likely inactivation mechanism by a visible USP laser. We have ruled out the following four possible inactivation mechanisms for non-enveloped, icosahedral viruses, namely: (1) inactivation due to ultraviolet C (UVC) photons produced by non-linear optical process of the intense, fundamental laser beam at 425 nm; (2) inactivation caused by thermal heating generated by direct laser absorption/heating of the virion; (3) inactivation resulting from a one-photon absorption process via chromophores such as porphyrin molecules, or indicator dyes, potentially producing reactive oxygen or other species; and (4) inactivation by the USP laser in which the extremely intense laser pulse produces shock wave-like vibrations upon impact with the viral particle. We present data which support a model whereby the inactivation mechanism for non-enveloped, icosahedral viruses is impulsive stimulated Raman scattering. This information will greatly aid our understanding of the structure of non-enveloped, icosahedral viruses.
**Introduction**

Potential contamination of pharmaceuticals, biologicals, and uncooked foods with viruses is a critical problem. Conventional disinfection methods have serious potential side effects; for example, biochemical and pharmaceutical disinfection methods involve adding potentially toxic or carcinogenic chemicals, such as detergents and photosensitizers which are impossible to remove completely after the treatments. In addition, the added chemicals may interact with the product itself, potentially altering its structure or function. Ionizing radiation such as ultraviolet and gamma rays can be used to sanitize foods and destroy viral pathogens in biologicals, but the amount of irradiation required for viruses is relatively high, potentially damaging biological products and cells and making this less desirable for foods due to consumer concern. Microwave absorption method is not viable because water usually coexists with biological systems, and water severely absorbs light in the microwave spectral range, leading to heating effects.

An ultrashort pulsed (USP) laser technology has recently been developed to circumvent these difficulties.\textsuperscript{1-10} The advantages of this novel technology are: (1) it is non-invasive; no foreign chemicals are added to the disinfection process, and therefore there is less concern for carcinogenic effects; (2) it does not use extremely high energy photons such as gamma or X-ray; as a result, no covalent or ionic bonds are broken and there is less chance of creating new, potentially toxic materials; (3) it specifically targets the capsid of a virus; therefore, drug-resistant, mutated strains of the pathogens can also be killed by the technology; and (4) it uses photons with a wavelength transparent to water; consequently, in contrast to the microwave absorption method, it does not cause heating effects.
Low-power ultrashort pulsed (USP) lasers operating at wavelengths of 425 nm and in the near-infrared region have been shown\textsuperscript{1-10} to effectively inactivate viruses such as human immunodeficiency virus (HIV), M13 bacteriophage, and murine cytomegalovirus (MCMV). It was shown previously\textsuperscript{1-5} that non-enveloped, helical viruses such as M13 bacteriophage were inactivated by a USP laser through an impulsive stimulated Raman scattering (ISRS) process. Recently, enveloped viruses like MCMV has been shown\textsuperscript{10} to be inactivated by a USP laser via protein aggregation induced by an ISRS process. However, the inactivation mechanism for a clinically important class of viruses – non-enveloped, icosahedral viruses – remains unknown.

There are a variety of possible inactivation mechanisms for non-enveloped, icosahedral viruses. These possibilities include: (1) inactivation due to ultraviolet C (UVC) photons produced by the intense laser beam through a non-linear optical process; (2) inactivation caused by thermal heating generated by direct laser absorption/heating of the virion; (3) inactivation resulting from a one-photon absorption process via chromophores such as porphyrin molecules or indicator dyes, potentially producing reactive oxygen or other species; and (4) inactivation via an ISRS process.

We note that there is one more possibility of inactivation by the USP laser in which the extremely intense laser pulse produces shock wave-like vibrations upon impact with the viral particle\textsuperscript{11}, leading to viral inactivation. However, this possibility can be ruled out because the laser intensity employed in our current laser experiments is too low to activate such an effect.

In this paper, we report examination of these possible inactivation mechanisms for a non-enveloped, icosahedral virus, namely murine norovirus-1 (MNV-1), and present evidence that supports ISRS as the most likely inactivation mechanism by a visible USP laser. MNV-1 was
chosen in this study because norovirus, which is highly contagious, is one of the leading viruses for food poisoning around the globe. This efficient, non-invasive approach for the eradication of non-enveloped, icosahedral viruses, when employed in a continuous, syringe-pumped configuration, can be applied to the disinfection of pathogens in pharmaceutical processes and the disinfection of blood products for transfusion.

**Materials and Methods**

**Virus stocks.** Working stocks of MNV-1 were prepared using confluent monolayers of mouse monocyte/macrophage RAW 264.7 cells (American Type Culture Collection, Manassas, VA) cultured in high glucose Dulbecco’s modified eagle media (DMEM; Gibco-Invitrogen Co., Grand Island, NY) without indicator supplemented with 25 mM HEPES buffer, 10% fetal bovine serum (FBS; Gibco-Invitrogen), 2 mM Gluta-MAX-1 (Gibco-Invitrogen), 100 U of penicillin, and 100 µg/ml of streptomycin sulfate (Gibco-Invitrogen), essentially as described by Wobus et al.\(^\text{12}\) Partial purification of MNV-1 was performed essentially as described by Lou et al.\(^\text{13}\) MNV-1 stocks were treated with 10 µg/ml of DNAse for 1 hr followed by addition of 1% lauryl sarcosine and 10 mM EDTA. Virus was pelleted by centrifugation at 82,000 × g for 6 h at 4°C in a TH-660 rotor (Sorvall). The pellet was resuspended in PBS and further purified by centrifugation at 175,000 × g for 6 h at 4°C using a sucrose step gradient of 10, 20, 30, 40 and 45% sucrose. The pellet was resuspended in Earle’s balanced salt solution (Gibco-Invitrogen) and dialyzed against Tris-buffer saline (20 mM Tris-HCl, pH 7.6, 0.14 M NaCl). MNV-1 samples were assayed using confluent 6-well dishes (Fisher Biotech, Fairlawn, NJ) inoculated
with 0.5 ml, or ten-fold serial dilutions prepared in Earle’s balanced salt solution (EBSS; Life Sciences), for 2 h at 37ºC followed by overlay with 2 ml of modified eagle media (Gibco-Invitrogen) containing 1.5% low melt agarose (Fisher Biotech) with 5% FBS, 2 mM Gluta-MAX-1, 100 U of penicillin and 100 µg/ml of streptomycin sulfate (Gibco-Invitrogen). After three days incubation, plaques were visualized by staining with 0.03% neutral red (Fisher Biotech) for 2 h at 37ºC. Because the 96-well tissue culture-treated flat bottom plates contain approximately 30,000 cells/well, depending upon the dilution of viral titer in the assaying process, the ratio of virus to cell can be 1.6 x 10³, 1.6 x10², 1.6 x10¹, 1.6, and 1.6 x 10¹.

**Laser treatment.** The excitation source employed in this work was a diode-pumped mode-locked Ti-sapphire laser. The laser produced a continuous train of 65fs pulses at a repetition rate of 80 MHz. The output of the second harmonic generation (SHG) system of the Ti-sapphire laser was used to irradiate the sample. The excitation laser was chosen to operate at a wavelength of 425 nm and with an average power as specified. It had a pulse width of full-width at half maximum (FWHM) of about 100 fs. An achromatic lens of focus length 5 cm was used to focus the laser beam into the sample area. In order to facilitate the interaction of laser with MNV-1, the viral sample with a volume of 0.1 ml in buffer solution was placed inside a Pyrex cuvette with a micromagnet stirring bar and stationed above a magnetic stirrer so that virions would be forced to enter the laser-focused volume. The titer of MNV-1 samples was 5×10⁷ PFU/ml. The assays were performed on the laser-treated samples after proper dilution. The typical exposure time of the sample to laser treatment was about 2 h. All the experimental results reported here were obtained at T = 25ºC and with the single-laser-beam excitation. Temperature increase of sample
solutions during USP laser treatments, as monitored by a thermocouple, did not exceed 2°C. The
tightest focused spot of the laser beam was approximately 100 nm and the average laser exposure
time of individual virion within the beam is estimated to be about 3.5 s. All the experiments were
carried out in triplicate. The errors were expressed in standard deviations (SD). Temperature was
measured during laser treatment experiments with a thermocouple.

Transmission Electron Microscopy. MNV-1 samples were visualized using 400 mesh, copper
grids (Ted Pella Inc., Redding, CA) coated with a 0.25% Formvar solution (Electron Microscopy
Sciences, Fort Washington, PA). Grids were negatively stained with 1% phosphotungstic acid
(Polysciences Inc., Warrington, PA), pH 7.0 and examined under a Philips CM12 transmission
electron microscope (Philips, Eindhoven, The Netherlands) at an accelerating voltage of 80 KV.
Images were collected with a 4000M-T1-GE-AMT detector (DVC Co., Austin, TX) and
processed with AMT V600 software (AMT, Danvers, MA).

Real-Time PCR measurements. Real-Time PCR measurements were performed on the genome of MNV-1 without the laser treatment (control) and after the laser treatment at an average laser power density of 100 MW/cm², following the standard procedures as described by the manufacturer, by using Applied Biosystems model 7500 fast Real-Time PCR system from life Technologies. For MNV-1, sense primer 6622 5′-CGCCTTTACCAATTGGCC-3′ and antisense primer 6875 5′-TGAAAGAGTTGTTGGAGC-3′ were used at an annealing temperature of 64 °C to produce a 273-bp amplicon. Reverse transcription of all viral RNA was performed at 50 °C for 30 min followed by a 15-min Taq activation step at 95 °C. Forty cycle PCR reactions
were performed using annealing times of 1 min, a 1-min extension step at 72 °C and a 30-s denaturation step at 95 °C. For the final cycle, the annealing time was extended to 2 min and the final extension was performed for 10 min. All primers were used at a final concentration of 0.1 μg/50 μl reaction mix or approximately 0.25 μM for each primer.

Results

Efficient inactivation of MNV-1 by USP laser treatment

Since virus stocks and samples are often propagated in complex tissue culture media that contains potentially chromogenic constituents such as neutral red indicator dye, amino acids, cellular proteins and nucleic acids, MNV-1 used in this work was propagated in indicator-free media and partially purified by sucrose gradient or Optiprep gradient ultracentrifugation, respectively. Figure 1(a) shows a bar graph of the plaque forming units (PFU) for control and laser-treated MNV-1 samples in buffer solution. The control represents the sample with no laser treatment. We observed a load reduction of 3.1±0.1 in log_{10} scale for the laser-treated MNV-1 group. Here, load reduction is defined as the ratio of the number of plaques in the control to the number of plaques in the laser-treated sample. For reference, Figure 1(b) shows a load reduction of 3.0±0.1 in log_{10} scale for unpurified laser-treated MNV-1. We observed that the temperature of laser-treated samples did not increase by more than 2°C above room temperature as monitored by a thermocouple.
Fig 1. (a) Graphs of plaque forming units (PFU)/ml for control (without laser treatment) and laser-treated MNV-1 purified samples. (b) Graphs of plaque forming units (PFU)/ml for control (without laser treatment) and laser-treated MNV-1 unpurified samples.

Laser power-density dependence of inactivation of MNV-1

To gain better insight into the mechanism of inactivation for these non-enveloped, icosahedral viruses by USP laser treatment, we measured the fraction of MNV-1 survival as a function of laser power density, which is shown in Figure 2 in natural logarithm (ln) scale. The laser exposure time was 2 hours. Here, the fraction of survival is defined as the reciprocal of the load reduction. The fraction of virus survival has been found to decrease as the laser power density increases in a continuous fashion up to about 80 MW/cm². As the laser power density increases beyond 80 MW/cm², the fraction of survival drops precipitously.
Fig 2. The fraction of survival as a function of laser power density for a MNV-1 sample. A rapid decrease in the fraction of survival has been found at a laser power density of around 80 MW/cm$^2$.

Transmission electron microscope images of laser-treated MNV-1

Typical transmission electron micrographs of the control and laser-treated MNV-1 samples are shown in Figure 3. The spherical structure of diameter $\approx 30$ nm in Figure 3(a) shows the presence a control MNV-1 particle. Figure 3(b) shows that, after laser treatment with a power density of $1.1 \pm 0.2$ MW/cm$^2$, the capsid of the inactivated MNV-1 becomes cracked, presumably along the weak structural links, but remains globally intact, as evidenced by the appearance of smaller structures of about 10 nm in diameter on the capsid (here, for the sake of clarity, only one inactivated MNV-1 is shown). This transmission electron microscope image clearly reveals the locations of weak structural links in the capsid of a non-enveloped,
icosahedral virus – MNV-1. Figure 3(c) shows that as the laser power density increases to $100 \pm 10 \text{MW/cm}^2$, the capsid of the inactivated MNV-1 becomes disintegrated and separated into small pieces of diameter about 10 nm.

**USP laser spectral-width dependence of MNV-1 inactivation**

The theory of ISRS developed for the single-pulse excitation configuration makes predictions that the energy contained within the spectral width of a USP laser must be larger than the energy of a given molecular vibration to excite that specific vibrational motion in the molecule\textsuperscript{15}. In order to test whether the inactivation is due to an ISRS process or not, inactivation experiments were also carried out as a function of the laser spectral width. The results are shown in Figure 4. Virus inactivation has been found to be very sensitive to the FWHM of the laser spectral width. Very limited or no inactivation was observed for laser spectral width $\leq 1 \text{cm}^{-1}$, while substantial inactivation was observed for laser spectral width $\geq 2 \text{cm}^{-1}$.
Fig 3. TEM images of MNV-1 (a) without laser treatment (the control); (b) with laser treatment at a power density of $1.1 \pm 0.2 \text{ MW/cm}^2$ (here, for the sake of clarity, only one inactivated MNV-1 is shown); (c) with laser treatment at a power density of $100 \pm 10 \text{ MW/cm}^2$. The spherical structure with a diameter of around 30 nm in (a) represents the presence of MNV-1 in the control. At the intermediate laser power density, (b) shows that the inactivated MNV-1 particle forms cracks at the structural links of the capsid. At the high laser power density, (c) demonstrates the disintegration of the capsid of the inactivated MNV-1 into spherical structures with a diameter of around 10 nm.
Fig 4. MNV-1 survival as a function of laser spectral width. The laser intensity is kept constant as shown. The fraction of survival decreases rapidly at a laser spectral width of around 1.0 cm$^{-1}$, consistent with the prediction by the impulsive stimulated Raman scattering process using a one laser configuration. Laser exposure time was 2 h. Error bars represent S.D.

Real-time PCR results on the genome of MNV-1 with and without USP laser treatment

In order to test the effects of USP laser treatment on the genome of MNV-1, Figure 5 shows the real-time PCR counts in linear scale for the genome of MNV-1 without (control) and with laser treatment at an average laser power density of 100 MW/cm$^2$. The similarity of real-time PCR counts between the control and laser-treated samples suggests that, within the amplicon size of 273 bp tested, there is very minimal genome degradation/damage for MNV-1 after the laser treatment. This result is consistent with our previous reports by using gel electrophoresis that USP laser irradiation under similar experimental conditions does not damage the genome of either M13 bacteriophage$^{6,8}$ or MCMV$^{10}$. 
Fig 5. Real-time PCR counts in linear scale for the genome of MNV-1 without (control) and with laser treatment at an average laser power density of 100 MW/cm². The similarity of real-time PCR counts between the control and laser-treated samples suggests that there is very minimal genome degradation/damage for MNV-1 after the laser treatment.

Discussion

As mentioned previously, potential mechanisms for the inactivation of non-enveloped, icosahedral viruses such as MNV-1 include: (i) the production of UVC photons by a non-linear optical process; (ii) thermal heating by direct laser absorption; (iii) a one-photon absorption process by chromophores, such as porphyrin molecules¹⁶ or indicator dyes producing reactive oxygen species or other reactive species; and (iv) the ISRS process. We now examine these possible mechanisms in detail.

Inactivation cannot be due to generation of UVC photons through non-linear optical processes

First, we have estimated the number of UVC photons which might be produced at laser power densities used in our experiments. The non-linear optical coefficients for murine norovirus
and human papillomavirus are not available in the literature; however, if we assume that these viruses are giant molecules and that their non-linear coefficients are comparable to that of a typical molecule\textsuperscript{17}, then the number of UVC photons generated under our experimental conditions is estimated to be of the order of 0.1 per second. The laser exposure time is two hours; the titer of viral particles is about 50 million; thus the value of generated UVC photons is too small to account for the load reduction observed in our inactivation experiments for MNV-1.

Secondly, we have tried to detect UVC photons which might be generated in our laser experiments (here, the glass vial is replaced by a synthetic fused silica vial) by using a UV spectrometer with a photon counting system. Within our experimental uncertainty of $\pm 1$ photon per second we failed to detect any UVC photons. This finding is consistent with our estimation stated above. Therefore, the observed inactivation for MNV-1 cannot be due to UVC photons generated by non-linear optical process.

**Inactivation is not due to thermal heating effects**

In order for the USP laser to thermally damage the virus, there must be direct energy transfer between the laser and viral particle. We observed that the temperature of laser-treated samples did not increase by more than 2°C above room temperature as monitored by a thermocouple. MNV-1 is known to be highly thermostable, and readily tolerant of temperatures in excess of 60°C\textsuperscript{18,19}. Therefore, heating of the entire sample, termed macro-thermal effects, can be ruled out as an explanation for inactivation.

However, we note that it is possible that laser treatment could create “micro-thermal” effects where the laser energy might be absorbed by the viral particle itself and spread
throughout the volume of the virus, heating up the viral particle and leading to the inactivation. In this micro-thermal scenario, one would expect that for a given laser power density, viruses with different sizes would be heated to different temperatures.

For icosahedral viruses, if we consider a simple model calculation in which the capsid of the virus is assumed to completely absorb the incident photons and the energy thermalization within the viral particle is significantly faster than that between the viral particle and its surroundings, the total deposited energy is expected to be proportional to the cross-sectional area of the virus. In the micro-heating scenario, this deposited energy spreads/thermalizes over the whole volume of virus and increases the temperature of the virus. The total laser energy deposited is given by

\[ E = I \cdot S = m \cdot c \cdot \Delta T = \rho \cdot V \cdot c \cdot \Delta T \quad \text{(1);} \]

where \( I \) is the laser intensity;

\( S \) is the cross-sectional area of the virus; \( m \) is the mass of the virus; \( c \) is the specific heat of the virus; \( \Delta T \) is the increase of temperature of the virus; \( \rho \) is the density of the virus; \( V \) is the volume of the virus.

For a given laser intensity \( I \), if \( \rho, c \) are assumed to be constant, because \( S \propto r^2 \) and \( V \propto r^3 \), we have

\[ \Delta T \propto \frac{1}{r} \quad \text{(2);} \]

where \( r \) is the radius of the virus.

Therefore, for a given laser intensity \( I \), the expected temperature increase (\( \Delta T \)) would be less for a larger virus than for a smaller virus.
When we compare load reduction of MNV-1 and HPV-16 experiments under the same laser parameters, we find that the load reduction of $3.1\pm0.1$ and $5.1\pm0.5$ in $\log_{10}$ scale for MNV-1 and HPV-16 (which is not shown here), respectively. The diameters of MNV-1 and HPV-16 virions are approximately 30 and 55 nm, respectively. Thus, the observation that the larger icosahedral virus is more readily inactivated, argues against a micro-thermal mechanism. We note that even though the energy transfer between the virion and its environment is taken into account, because a larger virus has a larger surface area, this results in greater energy loss, and thereby a lesser temperature increase ($\Delta T$), which again is in contradiction with our experimental observation of viral load reductions for MNV-1 and HPV-16.

**Inactivation is not due to one-photon absorption**

For this one-photon absorption scenario, chromophores that are present in the sample solution, upon absorption of laser of wavelength 425 nm, may produce reactive oxygen species, leading to the inactivation of viral particles. This possibility is unlikely for the following reasons: (1) we have not observed any detectable absorption by MNV-1 viral particles at a wavelength of 425 nm; (2) MNV-1 used in this work was propagated in indicator-free media and substantially purified by sucrose gradient and Optiprep gradient centrifugation respectively, which presumably removed extraneous cellular proteins which might absorb blue laser light; (3) we have tested and found that the load reduction for the purified MNV-1 samples was consistent with that for which MNV-1 samples were not purified; (4) we tested the inactivation of MNV-1 by the USP laser within orange juice as well as with apple juice spiked with MNV-1. Within the experimental uncertainty, the load reduction of MNV-1 in MNV-1-spiked orange/apple juice was
found to be the same as that in the buffer solution. Because both orange juice and apple juice are loaded with antioxidant – including vitamin C, which is able to efficiently scavenges reactive oxygen species – these experimental results further confirm that the inactivation of MNV-1 by the USP laser cannot be due to the production of reactive oxygen species through the one-photon absorption process.

Our experimental results are consistent with inactivation by an ISRS process

ISRS has been shown to be able to excite vibrational oscillations in solids and molecules\textsuperscript{15,20-26}. In general, these experiments have been performed by using two independently-tunable, USP lasers emitting separate and distinct wavelengths. However, in this work, we employed a special one-laser-beam excitation configuration originally described by Yan et al\textsuperscript{15}. The theory of the ISRS process under a one-laser-beam excitation configuration shows\textsuperscript{15,27} that when the laser intensity is kept constant, the amplitude of the laser excited vibrational motion is proportional to $e^{-\left(\frac{\omega_0^2}{\Delta\omega_L}\right)^2}$, where $\omega_0$ is the angular frequency of the excited vibrational motion and $\Delta\omega_L$ is the full-width-at-half-maximum (FWHM) of the laser spectral width. Because the excited amplitude of vibration depends on the factor $-\omega_0^2 / \Delta\omega_L^2$ in an exponential fashion, the ISRS process predicts that a threshold laser spectral width, which is comparable to the angular frequency of the global vibrational motion of the virus, exists for viral inactivation. Specifically, because the angular frequency of the global vibrational modes of an icosahedral virus with 30 nm in diameter is estimated to be about 2 cm$^{-1}$\textsuperscript{28}, the ISRS inactivation mechanism predicts that there exists a laser spectral-width threshold of about 1 cm$^{-1}$ for the
inactivation of MNV-1 by a USP laser. This prediction is indeed observed in our experiments as described below.

To verify this prediction of the ISRS inactivation mechanism, we have performed inactivation experiments for MNV-1 as a function of the laser spectral width, which is shown in Figure 5. If we take $\omega_0$ to be $2.0 \text{ cm}^{-1}$ for a non-enveloped, icosahedral virus with a diameter of 30 nm (29), $e^{-\left(\omega_0^2 / \Delta \omega_L^2\right) \cdot 4}$ has values of $8.9 \times 10^{-6}$, $1.8 \times 10^{-2}$, $3.7 \times 10^{-1}$, $7.7 \times 10^{-1}$, $8.9 \times 10^{-1}$, $9.4 \times 10^{-1}$ for $\Delta \omega_L = 0.25$, $0.50$, $1.0$, $2.0$, $3.0$, $4.0 \text{ cm}^{-1}$, respectively. In other words, the ISRS process predicts that in order to excite a sizable vibrational amplitude to break hydrogen bonds/ hydrophobic contacts and achieve inactivation, the laser spectral width has to be comparable to, or larger than, the angular frequency of the oscillations. This prediction is indeed in consistence with the experimental results of Figure 5 in which very little or no inactivation has been found when the laser spectral width $\Delta \omega_L$ is $\leq 0.5 \text{ cm}^{-1}$, while significant inactivation is found for $\Delta \omega_L \geq 2 \text{ cm}^{-1}$. Consequently, we attribute the inactivation mechanism of the non-enveloped, icosahedral MNV-1 by the USP laser to the ISRS process.

To explain the data for MNV-1, we divided the data into two parts: the first part consists of data prior to the occurrence of the sharp drop in the fraction of survival (i.e., for laser power density up to 75 MW/cm$^2$) and the second part consists of data within the observed sharp drop in the fraction of survival (power density $\geq 80$ MW/cm$^2$). We have found that the first part of the data can be fit reasonably well by the following function$^{29}$:

$$y = \exp(-Ax)$$

(3);
where \( y \) is the fraction of survival; \( x \) is the laser power density; and \( A \) is the effective inactivation rate constant. Figure 6 shows the best fit of our data, modeled as a solid line with

\[
A = (0.017 \pm 0.001) cm^2/W.
\]

**Fig 6.** The MNV-1 survival fraction as a function of laser power density, fit with Eq. (3) for laser power density \(< 80 MW/cm^2\) (solid line). Error bars represent S.D.

We did not attempt to fit the second part of the data with laser power density \( \geq 80 \) MW/cm\(^2\). However, the qualitative behavior, a sharp drop in the fraction of survival in this range of laser power densities, indicated that the effective inactivation rate in this range of laser power densities is much larger than that obtained from the first part of the data with laser power density up to 75 MW/cm\(^2\).
Considering the energy required to break hydrophobic contacts and hydrogen bonds, it is possible to explain the observed laser power density results shown in Figures 2 and 6. Two disparate inactivation curves were observed: one for laser power densities up to 75 MW/cm² and the other for laser power densities of 80 MW/cm² and above. We note that the energy of a typical covalent bond in a biological system is about 4 eV or larger. In our laser experiments, the relatively low photon energy of 2.92 eV generated from the USP laser employed at a wavelength of 425 nm means that weaker hydrogen bonds or hydrophobic contacts can be broken but not the stronger covalent/ionic bonds. Furthermore, the broken hydrogen bonds and hydrophobic contacts in a molecule have been demonstrated to reform on a time scale of about 10 picoseconds at room temperature. With this knowledge in mind, we are able to explain the relatively small effective inactivation rate constant $A$ (from equation (3)) deduced for laser power density of $\leq 75$ MW/cm². Because the laser power density is relatively small, the number of broken hydrogen bonds/hydrophobic contacts is relatively small; and since these broken bonds are rapidly reformed, the majority of the laser-treated viruses having broken bonds is expected to have their broken bonds reformed and restored to their non-irradiated structures. The capsid of a virus is an integral part in the viral infection process; any damage/alternation to its structure such as breaking of some hydrogen bonds or hydrophobic contacts can significantly affect its infectious capability. Therefore, it is the minority of laser-treated viruses, which have not been restored to their original structures, that contributes to the relatively smaller effective inactivation rate constant observed in this range of laser power densities. The laser power density of 75 MW/cm² simply reflects the threshold laser power density in which the number of broken bonds that can be easily reformed and the structure of the virus can be easily restored for MNV-1. On the other
hand, at the laser power density of $\geq 80 \text{ MW/cm}^2$, inactivation for MNV-1 was dramatically enhanced, as indicated by the sharp drop in the fraction of survival in this range of laser power density. We interpret this to be reminiscent of a deposited laser energy threshold through the ISRS process that once exceeded, results in so many simultaneously broken hydrogen bonds and hydrophobic contacts that the entire capsid is disintegrated spatially (as shown in TEM image of Figure 3(c)); as a result, reformation of bonds and restoration to the original viral structure is greatly reduced. Therefore, at the laser power density of $\geq 80 \text{ MW/cm}^2$, the capsid of MNV-1 begins to disassemble and the effective inactivation rate increases dramatically. It is worthwhile mentioning that the TEM images shown in Figure 3 not only provide direct experimental evidence of the disruption of the capsid in a non-enveloped, icosahedral virus by USP laser irradiation but also reveal the locations of weak structural links on its capsid. This important information can help understand the fundamental structure of non-enveloped, icosahedral viruses.

We note that in the previous studies of inactivation of enveloped virus like MCMV\textsuperscript{10}, the global structure of the non-irradiated and USP laser-inactivated viruses are almost identical. However, the protein gel electrophoresis experiments indicated that the inactivation was due to protein aggregation within the virion, induced by the ISRS process. The aggregate was identified to be composed of capsid protein and tegument protein by mass spectrometry analysis. For MCMV, the icosahedral nucleocapsid is surrounded by tegument protein and a lipid envelope through which a number of glycoproteins protrude. We believe the reason why the MCMV capsid remained intact after the USP laser inactivation is most likely because of significant
damping produced on the capsid of MCMV by the surrounding amorphous tegument and lipid envelope layers.

In conclusion, we have investigated the inactivation mechanism of a non-enveloped, icosahedral virus, MNV-1, by USP laser irradiation. Possible mechanisms of inactivation were thoroughly examined. Real-time PCR measurements indicate that, within the amplicon size of 273 bp tested, USP laser irradiation does not degrade the genome of MNV-1. We conclude that our model non-enveloped virus, MNV-1, is inactivated by the ISRS process. These studies provide fundamental knowledge on photon-virus interactions on femtosecond time scales. From the analysis of the TEM images of viral particles before and after USP laser irradiation, the locations of weak structural links on the capsid of MNV-1 were revealed. This important information will greatly aid our understanding of the structure of non-enveloped, icosahedral viruses. We envision that this non-invasive, efficient viral inactivation method will find applications in the disinfection of pharmaceuticals, biologicals, and blood products in the near future. This novel, unique approach for the eradication of non-enveloped, icosahedral viruses, when employed in a continuous, syringe-pumped configuration, can be applied to the disinfection of pathogens in pharmaceutical processes and to the disinfection of blood products for transfusion.

**Acknowledgements**

The authors thank Gloria Meade (USDA Dover) for technical assistance, and Arisitides Marcano (Delaware State Univ., Dover, DE) Gary Richards (USDA Dover, DE), and Chao Chen (Indiana Univ, Bloomington IN) for technical review of this manuscript. This work was
supported by intramural funding from the United States Department of Agriculture, Agricultural Research Service and NHLBI Ruth L. Kirschstein NRSA F30 grant HL116183-01 (Shaw-Wei D. Tsen).

References

CHAPTER 4

Ultrashort pulsed laser treatment inactivates viruses by inhibiting viral replication and transcription in the host nucleus.
Chapter 4 is a reformatted version of a published manuscript: “Shaw-Wei D. Tsen, Travis Chapa, Wandy Beatty, et al. Ultrashort pulsed laser treatment inactivates viruses by inhibiting viral replication and transcription in the host nucleus. Antiviral Research (in press, doi: 10.1016/j.antiviral.2014.07.012)”. Under Dr. Samuel Achilefu’s supervision, my contributions to this work included designing and developing the ultrashort pulsed laser technology, carrying out the characterization and laser inactivation experiments, interpreting and analyzing data, and writing the manuscript. In this Chapter, I discuss my work in the characterization of the functional aspects of ultrashort pulsed laser-inactivated viruses in vitro.
Abstract

Ultrashort pulsed laser irradiation is a new method for virus reduction in pharmaceuticals and blood products. Current evidence suggests that ultrashort pulsed laser irradiation inactivates viruses through an impulsive stimulated Raman scattering process, resulting in aggregation of viral capsid proteins. However, the specific functional defect(s) in viruses inactivated in this manner have not been demonstrated. This information is critical for the optimization and the extension of this treatment platform to other applications. Toward this goal, we investigated whether viral internalization, replication, or gene expression in cells were altered by ultrashort pulsed laser irradiation. Murine Cytomegalovirus (MCMV), an enveloped DNA virus, was used as a model virus. Using electron and fluorescence microscopy, we found that laser-treated MCMV virions successfully internalized in cells, as evidenced by the detection of intracellular virions, which was confirmed by the detection of intracellular viral DNA via PCR. Although the viral DNA itself remained polymerase-amplifiable after laser treatment, no viral replication or gene expression was observed in cells infected with laser-treated virus. These results, along with evidence from previous studies, support a model whereby the laser treatment stabilizes the capsid, which inhibits capsid uncoating within cells. By targeting the mechanical properties of viral capsids, ultrashort pulsed laser treatment represents a unique potential strategy to overcome viral mutational escape, with implications for combatting emerging or drug-resistant pathogens.
Introduction

Ultrashort pulsed (USP) lasers are an attractive, chemical-free method to inactivate pathogens in pharmaceuticals and blood products\(^1\). Visible USP laser irradiation is a unique strategy to overcome the limitations of conventional antiviral agents due to its physical mechanism of action – the excitation of molecular vibrations within viral capsids through impulsive stimulated Raman scattering (ISRS)\(^2\). This strategy targets fundamental mechanical properties of viruses that may be difficult to escape through genetic mutation. Additionally, visible light shows negligible intrinsic absorption by nucleic acids and proteins in the absence of chromophores. Furthermore, unlike ultraviolet (UV) or gamma radiation, visible light does not cause molecular ionization. These properties could enable the use of USP lasers to selectively inactivate pathogens without harming desired biological materials such as mammalian proteins in blood products. Visible USP lasers have shown broad spectrum efficacy against both DNA and RNA viruses\(^2-9\), including non-enveloped viruses that are conventionally difficult to inactivate. Under these laser treatment conditions, the structure of a mammalian protein was well preserved\(^7\).

A previous study demonstrated the effects of visible USP laser irradiation on the molecular structure of Murine Cytomegalovirus (MCMV), an enveloped DNA virus\(^9\). This work showed that USP laser treatment causes aggregation of viral capsid proteins. It was proposed that the laser treatment, through ISRS, transiently induces partial unfolding of tertiary protein structures leading to density-dependent protein aggregation through exposed hydrophobic regions of proteins (Figure 1). Under this model, the high density capsid proteins of virions are
more susceptible to laser-induced aggregation relative to free proteins in solution. The study demonstrated that the laser-induced aggregation effect increased with the concentration of a purified protein.  

**Fig 1.** Proposed model for USP laser-induced protein aggregation. USP laser light scattering leads to transient, partial unfolding of protein structures, due to disruption of electrostatic interactions through ISRS. In cases where the protein is at high density, aggregation will occur between exposed hydrophobic patches on nearby proteins. In cases where the protein is at low density, the proteins rapidly recover by regaining their native conformation. Filled spheres indicate hydrophilic regions of the protein; hollow spheres indicate hydrophobic regions of the protein.

To date, however, functional studies of USP laser-treated viruses in cells are lacking. These data are needed to establish the molecular and functional basis for the abovementioned mechanistic hypotheses. In addition, such data should prove valuable for the optimization of the treatment process and for the extension of the technology to other applications. To this end, we
have investigated the effects of visible USP laser treatment on the viral trafficking and functional state in cells.

**Methods**

**Cells and viruses.** MEF 10.1 murine embryonic fibroblast cells\(^{10}\) were cultured in Dulbecco’s Modified Eagle Medium (DMEM), supplemented with 10% fetal calf serum, 1mM sodium pyruvate, and nonessential amino acids. Balb/3T3 murine embryonic fibroblast cells were cultured in RPMI medium, supplemented with 10% fetal calf serum and antibiotics. GFP-expressing MCMV virus (hereafter referred to as “MCMV”) was generated as previously described\(^{11,12}\). To produce viral stocks, MEF 10.1 cells were infected with MCMV at a low multiplicity of infection. Cell supernatants were harvested 5 d post-infection after 100% cytopathic effect and cleared of cell debris by centrifugation. Extracellular virions were pelleted by ultracentrifugation with sorbitol cushion and resuspended in phosphate-buffered saline (PBS). Viral titers were determined using a median tissue culture infectious dose (TCID\(_{50}\)) assay, as described below.

**Femtosecond laser irradiation.** The excitation source employed in this work was a diode-pumped continuous wave mode-locked Ti-sapphire laser. The laser produced a continuous train of 60 fs pulses at a repetition rate of 80 MHz. The output of the second harmonic generation
(SHG) system of the Ti-sapphire laser was used to irradiate the sample. The excitation laser was chosen to operate at a wavelength of $\lambda = 425$ nm and with an average power of approximately 120 mW. It has a pulse width of full-width at half maximum $= 100$ fs. A lens was used to focus the laser beam into a spot within the sample volume. MCMV virus was irradiated at a final concentration of about $5 \times 10^6$ TCID$_{50}$/ml. A magnetic stirring device was used to facilitate exposure of the sample to the laser beam. Irradiation was carried out at 22°C and with the single laser beam excitation. After laser irradiation, samples were immediately stored at -80°C.

**TCID$_{50}$ assays.** TCID$_{50}$ assays were performed to determine reduction in viral titers following laser irradiation. MEF 10.1 cells were seeded into 96 well plates at a density of $6 \times 10^4$ cells/mL and incubated overnight. Cells were approximately 100% confluent at the time of infection. Control (untreated) or laser-treated viruses were serially diluted and added to cells, which were incubated for 4 days. Viral titers were determined on day 4 post-infection by scoring each well for GFP-positive cells using a fluorescent microscope.

**Fluorescence imaging.** For tracking of viral internalization, laser-treated or control MCMV virions were labeled with PKH26 dye (Sigma) according to the manufacturer’s instructions. Balb/3T3 cells were infected with PKH26-labeled MCMV at a multiplicity of infection (MOI) of ~ 100 TCID$_{50}$/cell for 2 h, washed three times in PBS, and fixed with Vectashield mounting medium with DAPI (Vector Laboratories, Inc). For the time course imaging of viral GFP expression, Balb/3T3 cells were infected with control or laser-treated MCMV at an MOI of ~100
TCID_{50}/cell and imaged at 24 h, 48 h, and 72 h post-infection. Samples were visualized with a Zeiss Axioskop 2 Mot Plus fluorescence microscope equipped with an Axiocam MRm monochrome camera and a 10X, 0.3 numerical aperture Zeiss Plan Neo-Fluar objective or a 63X, 1.4 numerical aperture Zeiss Plan Apochromat oil objective. Images were acquired using Axiovision 4.6 software (Carl Zeiss Inc., Thornwood, NY).

**Electron microscopy.** MEF 10.1 cells were infected with control or laser-treated MCMV at an MOI of ~20 TCID_{50}/cell for 2 h. For ultrastructural analysis, infected cells were fixed in 2% paraformaldehyde/2.5% glutaraldehyde (Polysciences Inc., Warrington, PA) in 100 mM cacodylate buffer, pH 7.2 for 1 h at room temperature. Samples were washed in cacodylate buffer and postfixed in 1% osmium tetroxide (Polysciences Inc.)/1.5% potassium ferricyanide (Sigma, St Louis, MO) for 1 h. Samples were then rinsed extensively in dH2O prior to en bloc staining with 1% aqueous uranyl acetate (Ted Pella Inc., Redding, CA) for 1 h. Following several rinses in dH2O, samples were dehydrated in a graded series of ethanol and embedded in Eponate 12 resin (Ted Pella Inc). Sections of 95 nm were cut with a Leica Ultracut UCT ultramicrotome (Leica Microsystems Inc., Bannockburn, IL), stained with uranyl acetate and lead citrate, and viewed on a JEOL 1200 EX transmission electron microscope (JEOL USA Inc., Peabody, MA) equipped with an AMT 8 megapixel digital camera (Advanced Microscopy Techniques, Woburn, MA).
**PCR.** Intracellular viral DNA within MCMV-infected cells and virion-associated DNA were quantified by PCR amplification followed by agarose gel analysis. For detection of intracellular viral DNA, cells were infected with either control or laser-treated MCMV for 18 h. Cells were then washed in PBS, trypsinized, washed again in PBS, pelleted by centrifugation, and lysed. Cell lysates were used directly as PCR template. For detection of virion-associated DNA, virions were mixed in lysis buffer and either pre-digested with proteinase K for 2 h at 37°C or used directly as PCR template. In all PCR experiments, a primer pair specific for the MCMV IE1 gene was used\(^\text{11}\) (forward \(5'\) CAGGTTGGATCATGAAGCCT \(3'\), reverse \(5'\) AGCGCATCGAAAGACACG \(3'\)). 40 cycles were used with annealing time 1 min, extension step 1 min (72 °C) and denaturation step 30 s (95 °C). Gels were stained with SYBR Safe Green (Invitrogen) and visualized with a Kodak Multispectral Imaging System (Eastman Kodak).

**Western blotting analysis.** For detection of IE1 protein in MCMV-infected samples, MEF 10.1 cells were either mock-infected or infected with control or laser-treated MCMV at an MOI of \(~20\) TCID\(_{50}/\text{cell}\) for 4 h, 12 h, or 24 h. Samples were lysed, vortexed, and boiled for 5 min. Cell lysates were separated by SDS-PAGE and transferred onto nitrocellulose filters. Filters were incubated with anti-MCMV IE1 monoclonal antibody (CROMA101) (a generous gift from Stipan Jonjic, University of Rijeka, Croatia) or anti-mouse actin monoclonal antibody (Sigma). As a secondary antibody, horseradish peroxidase-conjugated anti-mouse IgG (Sigma) was used. Blots were developed by autoradiography.
Results

USP laser-treated virus is internalized by host cells

For all experiments, we used a GFP-expressing MCMV described in a previous report\textsuperscript{9,12}. Samples of MCMV were irradiated using a 425nm femtosecond laser. Laser-inactivated virus showed an approximate 5-log reduction in viral titers by TCID\textsubscript{50} assay, to a level near the limit of detection (Supplementary Figure 1). To determine whether virions were internalized by host cells, we used electron microscopy to view viral particles within murine embryonic fibroblast cells that had been infected with control (untreated) MCMV or USP laser-treated MCMV for 2 h. In both control and laser-treated groups, we observed the presence of MCMV virions within cells (Figure 2). To confirm these findings, we labeled control or laser-treated MCMV with a fluorescent dye, PKH26. Murine fibroblast cells were infected with PKH26-labeled control or laser-treated MCMV for 2 h before imaging by fluorescence microscopy. In both control and laser-treated groups, we found fluorescent virus particles within cells (Figure 3). These results were further confirmed by PCR detection of MCMV genomic DNA in cells infected with both control and laser-treated MCMV (Figure 4). Therefore, laser treatment does not alter the ability of MCMV virions to be internalized by cells.
Fig 2. Electron microscopy shows cellular internalization of USP laser-treated MCMV. Control or laser-treated MCMV were infected into murine embryonic fibroblast cells, and the cells were harvested, fixed, and sectioned for imaging. Images show cellular internalization of control MCMV or USP laser-treated MCMV at 2h post-infection. Arrows indicate intracellular virions.

Fig 3. Fluorescence microscopy shows cellular internalization of USP laser-treated MCMV. PKH26-labeled control or laser-treated MCMV were infected into murine embryonic fibroblast
cells. Images show combined bright field and fluorescence depicting cellular internalization of control MCMV or USP laser-treated MCMV at 2h post-infection.

Fig 4. MCMV DNA is present in cells infected with USP laser-treated MCMV. Murine embryonic fibroblast cells were infected with control or laser-treated MCMV. PCR analysis was performed at 18h post-infection with primers specific for the MCMV IE1 gene. M: molecular weight DNA ladder; 1: cells infected with control MCMV; 2: cells infected with laser-treated MCMV.

USP laser-treated virus cannot express virus-encoded genes in cells

Fig 4. MCMV DNA is present in cells infected with USP laser-treated MCMV. Murine embryonic fibroblast cells were infected with control or laser-treated MCMV. PCR analysis was performed at 18h post-infection with primers specific for the MCMV IE1 gene. M: molecular weight DNA ladder; 1: cells infected with control MCMV; 2: cells infected with laser-treated MCMV.

USP laser-treated virus cannot express virus-encoded genes in cells

We used GFP-expressing MCMV as an indicator of viral DNA replication in host cells. MCMV replicates and produces progeny virus over the course of 18-36 h post-infection\textsuperscript{13,14}. To assess the replication of MCMV in host cells, murine fibroblast cells were infected with either control or laser-treated MCMV and imaged for GFP fluorescence at 24, 48, and 72h post-infection (Figure 5). In cells infected with control MCMV, we observed strong GFP signal in the majority of cells at 24, 48, and 72 h (in addition to cytopathic effect). In contrast, cells infected with laser-treated MCMV did not exhibit observable GFP signal even after 72 h post-infection.

83
This result demonstrates that laser-treated MCMV virus does not replicate when infected into cells.

![Figure 5](image)

**Fig 5.** USP laser-treated MCMV cannot replicate in cells. Control GFP-expressing MCMV or laser-treated GFP-expressing MCMV virus were infected into murine embryonic fibroblast cells and imaged for GFP expression 24, 48, and 72h post-infection using fluorescence microscopy. Images show GFP expression in cells infected with either control MCMV or laser-treated MCMV at 24, 48, and 72h post-infection. BF, bright field; GFP, green fluorescent protein.

To further confirm the effect of USP laser treatment on viral gene expression, we assessed the expression of the MCMV immediate early 1 (IE1) protein within infected cells, which is one of the initial viral proteins expressed by the cell upon MCMV infection\textsuperscript{13,14}. We infected murine fibroblast cells with control or laser-treated MCMV virions for 4 h, 12 h, or 24 h, and the cell lysates were then immunoblotted for IE1 (Figure 6). We observed expression of IE1 protein in cells infected with control MCMV at all three time points tested. In contrast, we did not detect any IE1 protein in cells infected with laser-treated MCMV. Since IE1 is an
important gene that activates downstream virus transcription, these results suggest that many MCMV genes are likely not expressed during infection with laser-treated virus.

**Fig 6.** IE1 protein is absent in cells infected with USP laser-treated MCMV. Murine embryonic fibroblasts were either mock-infected or infected with control (untreated) MCMV or laser-treated MCMV. At the time points after infection indicated, cells were harvested and lysed, subjected to SDS-PAGE electrophoresis analysis, and immunoblotted with anti-IE1 or anti-actin monoclonal antibodies.

Based on our observations, there was the possibility that the USP laser treatment may be damaging the MCMV genomic DNA. However, this effect is unlikely because USP laser treatment at 425 nm lacks sufficient energy to disrupt covalent bonds in DNA. A previous study also failed to detect any effect of USP laser treatment on MCMV DNA. To provide further evidence that USP laser treatment does not damage MCMV genomic DNA, we showed that the MCMV IE1 gene can be PCR-amplified from both control and laser-treated MCMV virions (Figure 7). Therefore, the MCMV DNA remains sufficiently intact to be polymerase-amplifiable (i.e., it can still be utilized as a template for DNA polymerases) after laser treatment. These
results indicate that in the context of cellular infection, the functional defect of the laser-treated virus lies upstream of viral gene expression.

**Fig 7.** DNA from USP laser-treated MCMV virions is PCR-amplifiable. Control or laser-treated MCMV virions were subjected to PCR analysis with primers specific for the MCMV IE1 gene. M: molecular weight DNA marker; 1: control MCMV; 2: laser-treated MCMV; 3: control MCMV pre-digested with proteinase K; 4: laser-treated MCMV pre-digested with proteinase K.

**Discussion**

In a previous study, USP laser irradiation was shown to cause the aggregation of MCMV capsid proteins, resulting in stabilization of the viral capsid against detergent dissociation. In this report, we found indirect evidence for a functional defect in capsid function of USP laser-treated virions. Our data indicate that the defect in USP laser-treated virus occurs downstream of
cell internalization but upstream of viral replication/gene expression. The results support a model whereby the USP laser treatment aggregates the viral capsid, thus inhibiting intracellular capsid uncoating and preventing the viral DNA from undergoing replication or transcription in the host cell nucleus. The precise mechanisms governing capsid uncoating in MCMV are poorly understood, thereby hampering more detailed analyses of the behavior of USP laser-treated capsids in cells. A likely scenario is one whereby the virus is defective in either intracellular capsid uncoating or translocation. The previous report on USP laser-induced aggregation of viral capsid proteins\textsuperscript{9} suggests that the laser may stabilize the viral capsid, preventing it from disassembling properly within cells and thus “trapping” viral DNA so that it is unable to be replicated or transcribed in the nucleus (Figure 8). Based on the available evidence, this model provides the most plausible functional explanation for the USP laser inactivation of viruses. It is worth noting that in certain herpes viruses, the minor capsid protein UL25 is required for viral DNA release at the nuclear pore\textsuperscript{15}. A previous study showed that MCMV UL25 was among the proteins that were affected in USP laser-treated MCMV virions\textsuperscript{9}. Therefore, in addition to the resistance of aggregated capsid complexes to dissociation, laser-induced damage to UL25 might also contribute to impaired capsid uncoating.

While many physical methods such as ultraviolet (UV) light and gamma radiation cause covalent damage to proteins and nucleic acids, visible USP laser irradiation lacks the energy to perturb covalent structures in viruses. Instead, the USP laser treatment relies on disruption of noncovalent electrostatic interactions within and between proteins, leading to density-dependent aggregation of capsid proteins through ISRS to inactivate viruses\textsuperscript{9}. Unlike heating (which causes widespread denaturing of proteins), the postulated USP laser mechanism is expected to damage
proteins in high-density microenvironments while leaving other proteins relatively unaffected. We note that both heat and UV treatment lead to extensive denaturation of proteins, and therefore these methods cannot be used for sterilization of biologicals. On the other hand, we have demonstrated that the USP laser technique preserves the structure of proteins\(^7\). Therefore, in contrast to heat and UV techniques, the USP laser method is novel and has applications in pathogen reduction of blood products and other biologicals.

Fig 8. Proposed model for USP laser-induced viral capsid defect. USP laser treatment causes aggregation of viral capsid proteins\(^9\). The laser-treated virus is internalized by cells, but the aggregated viral capsid cannot uncoat and thus the viral genome remains “trapped” and cannot replicate or express viral genes.
We note that at this time the precise mechanism of MCMV entry into cells, including critical receptor(s) involved, is not entirely clear. Therefore, a receptor blocking study was not feasible. However, we have previously shown that the structure of proteins such as bovine serum albumin is retained after laser treatment\textsuperscript{7}. Along with these data, the cellular internalization studies in this report support a model in which laser treatment does not alter the structure of viral envelope proteins, and thus does not inhibit viral entry.

The results of this study also indicate a potential application of the USP laser method in vaccine production. The data suggest that USP laser-inactivated viruses retain their ability to enter cells by the normal infection route. If this is the case, then a USP laser-inactivated virus vaccine might be capable of eliciting the strong immune responses normally associated with live vaccines, but with the safety profile of a killed vaccine. Furthermore, as USP laser treatment preserves the structure of proteins, a vaccine prepared by USP laser treatment may lead to improved antibody responses compared to vaccines treated with heat or UV, which cause extensive protein damage.
Supplementary Fig 1. Virus inactivation efficiency. The titers of control or laser-treated MCMV samples were determined by TCID<sub>50</sub> assay.

Specific targeting of viral capsids through ISRS would make the USP laser technology unique among physical pathogen inactivation methods. From an evolutionary standpoint it is difficult to envision how viruses could alter fundamental aspects of their structure, namely the capsid protein density and/or vibrational frequencies of their capsids, through genetic mutation. Therefore, the USP laser method may be effective against newly emerging or rapidly mutating viral pathogens. In addition, the capsid is a universal feature of viruses. This would explain the broad spectrum virus inactivation which has been observed for USP lasers<sup>2-9</sup>. Finally, USP laser irradiation may be an effective way to generate whole virus vaccines with preserved antigenic/immunogenic structures.

To date, the functional aspects of USP laser-inactivated viruses have not been fully explored. The data presented in this manuscript are critical for the optimization and eventual
clinical translation of the technology. In addition, the functional studies in this report have yielded considerable insight toward the possible application of the laser technology towards vaccine development. Therefore, virus inactivation by USP lasers represents a unique and significant new field of study that warrants further investigation.

In conclusion, we reveal evidence for a functional capsid defect in USP laser-inactivated virus. This information will be indispensable for the translation of this platform technology toward clinical applications. Development of this USP laser technology for pathogen reduction of pharmaceuticals, antiviral therapies, and viral vaccine development is warranted.

**Acknowledgments**

This work was supported in part by the Mallinckrodt Institute of Radiology Development Fund, NIH grants R01 EB008111, R33 CA123537 (SA), and NHLBI Ruth L. Kirschstein NRSA F30 grant HL116183-01 (SDT).

**References**


CHAPTER 5

Pathogen Reduction in Human Plasma Using an Ultrashort Pulsed Laser
Chapter 5 is a reformatted version of a submitted manuscript: “Shaw-Wei D. Tsen, David H. Kingsley, Karen Kibler, et al. Pathogen reduction in human plasma using an ultrashort pulsed laser (manuscript submitted).” Under Dr. Samuel Achilefu’s supervision, my contributions to this work included designing and developing the ultrashort pulsed laser technology, carrying out the characterization and laser inactivation experiments, interpreting and analyzing data, and writing the manuscript. In this Chapter, I discuss my work in the demonstration of a novel application, pathogen reduction of human plasma, using the ultrashort pulsed laser technology.
Abstract

Pathogen reduction is an ideal approach to ensure the continued safety of the blood supply against emerging pathogens. However, the currently licensed pathogen reduction techniques are ineffective against non-enveloped viruses, and they introduce chemicals with concerns of side effects which prevents their widespread use. In this report we demonstrate the inactivation of both enveloped and nonenveloped viruses using a chemical-free method, a visible ultrashort pulsed laser. Laser treatment resulted in clinically relevant reductions in human immunodeficiency virus, hepatitis A virus, and murine cytomegalovirus in human plasma. Laser-treated plasma showed good retention of coagulation factors at a level comparable to other pathogen reduction methods currently in use. These results highlight the potential of ultrashort pulsed lasers as a method for chemical-free, broad-spectrum pathogen reduction in human plasma.
**Introduction**

Pathogen reduction (PR) is an ideal strategy to combat emerging pathogens and ensure the continued safety of blood products. However, the PR techniques that are currently in use for clinical blood products have limitations that preclude their widespread use. The solvent-detergent (SD) method, which inactivates enveloped viruses by disrupting lipid membranes, was discontinued in the United States due to an association with unexpected thromboses in some patients\(^1,2\). Light-activated photochemicals such as methylene blue and amotosalen, which inactivate pathogens by causing the formation of crosslinks between nucleic acids, also involve introducing chemicals with concerns of side effects\(^3\). Furthermore, all of the above mentioned methods are ineffective against non-enveloped viruses such as hepatitis A virus (HAV)\(^2\).

Ultrasound pulsed (USP) lasers have recently emerged as an attractive potential technique for pathogen inactivation\(^4\). The visible USP laser PR technology does not involve adding potentially toxic or carcinogenic chemicals. Visible USP lasers are non-ionizing and do not covalently modify proteins, thereby reducing concerns of neoantigen formation. Furthermore, USP laser treatment can inactivate a spectrum of viruses and bacteria\(^5-12\), including non-enveloped viruses that are traditionally difficult to inactivate. Under these treatment conditions, the structure of bovine serum albumin protein was well preserved\(^10\).

In this work we demonstrate inactivation of human immunodeficiency virus (HIV), HAV, and murine cytomegalovirus (MCMV) in human plasma using a USP laser operating at a wavelength of 425 nm. These pathogens are structurally representative of a broad range of viruses including enveloped RNA virus (HIV), non-enveloped RNA virus (HAV), and enveloped
DNA virus (MCMV). Furthermore, we assess the function of coagulation factors in treated plasma versus untreated plasma after USP laser irradiation.

**Methods**

**Femtosecond laser irradiation.** The excitation source employed in this work was a diode-pumped cw mode-locked Ti-sapphire laser. The laser produced a continuous train of 60 fs pulses at a repetition rate of 80 MHz. The output of the second harmonic generation (SHG) system of the Ti-sapphire laser was used to irradiate the sample. The excitation laser was chosen to operate at a wavelength of $\lambda = 425$ nm and with an average power of approximately 120 mW. It has a pulse width of full-width at half maximum (FWHM) = 100 fs. A lens was used to focus the laser beam into a spot within the sample volume. Samples were laser-irradiated for 90 min. A magnetic stirring device was used to facilitate exposure of the sample to the laser beam. Irradiation was carried out at 22°C and with the single laser beam excitation. After laser irradiation, samples were immediately stored at -80°C.

**Viruses and infectivity assays.**

**HIV inactivation.** HIV stock was propagated in MT-4 cells following transfection of HeLa cells with pNL4-3 plasmid (both MT-4 cells an pNL4-3 were kind gifts of Dr. Kuan-Teh Jeang).
Approximately $4 \times 10^6$ reverse transcriptase (RT) units of NL4-3 stock in 800 µl was added to 800 µl pooled normal plasma (George King Biomedical, Inc., Overland Park, KS). 200 µl was placed into each of 6 glass vials for transport to the laser lab. The 3 vials to be treated by the laser each contained a stir bar. Each of 3 vials was laser-irradiated as described above. During the treatment time, a vial serving as a room temperature control was placed into a beaker in the same room. During each treatment time, 4 of the 6 vials remained on ice. When each of 3 vials had been irradiated, all 6 vials were transported back to the HIV lab, where they were stored at -80°C until used in a MAGI assay. For the MAGI assay, the MAGI cells were obtained through the NIH AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH: U373-MAGI-CXCR4 CEM, catalog #3596 (contributed by Dr. Michael Emerman). The standard MAGI assay protocol was followed. Briefly, plasma/virus mixture was added to MAGI cells, incubated for 48 h, fixed, and X-gal staining was visualized. Three irradiated samples, three room temperature control samples, and the original virus stock were used in the assay. The final dilution factor for treated samples and controls was 1:2 compared to the original virus stock dilution used in the assay.

HAV inactivation. HAV was obtained from the American Type Culture Collection (Manassas, VA) as VR1402, a cell culture-adapted cytopathic clone of strain HM-175/18f. The virus was propagated on fetal rhesus monkey kidney (FRhK-4) cells. The HAV stock was stored at -70°C in DMEM (Gibco, Grand Island NY) with 10% FBS (Gibco) prior to use. To partially purify HAV, the virus was pelleted at 490,000 × g for 6 h, followed by resuspension of the HAV in
human plasma (George King Biomedical Inc., Overland Park, KS) and filtration through a 0.1 µm filter. Samples of human plasma alone or human plasma spiked with HAV were laser-irradiated as described above. Plaque assay was performed by making an initial 100-fold dilution followed by 10-fold serial dilutions made in Earle’s balanced salt solution (Gibco) and infecting 100-mm dishes confluent with FRhK-4 cell and infecting with 2 ml of virus dilution as described previously\textsuperscript{13}. After 2 h, the plates were overlaid with DMEM medium with 5% fetal bovine sera, and 1% agarose (Sigma-Aldrich, St. Louis, MO). At 17 days post-inoculation, HAV was inactivated by 10% formaldehyde treatment, the agarose overlay was removed, and HAV plaques were visualized by crystal violet staining (Fisher Scientific, Kalamazoo, MI).

\textit{MCMV inactivation.} Murine embryonic fibroblast 10.1 (MEF 10.1) cells were cultured in Dulbecco’s Modified Eagle Medium (DMEM), supplemented with 10% fetal calf serum, 1mM sodium pyruvate, and nonessential amino acids. GFP-expressing MCMV virus (hereafter referred to as “MCMV”) was generated as previously described\textsuperscript{14}. To produce viral stocks, MEF 10.1 cells were infected with MCMV at a low multiplicity of infection. Cell supernatants were harvested 24 h post-infection after 100% cytopathic effect and cleared of cell debris by centrifugation. Extracellular virions were pelleted by ultracentrifugation with sorbitol cushion and resuspended in phosphate-buffered saline (PBS). Samples of human plasma alone or human plasma spiked with MCMV were laser-irradiated as described above. Viral titers were determined using a median tissue culture infectious dose (TCID\textsubscript{50}) assay, as previously described\textsuperscript{12}. Briefly, MEF 10.1 cells were seeded into 96-well plates at a density of $1.25 \times 10^5$
cells/ml and incubated overnight. Cells were approximately 80% confluent at the time of infection. Laser-treated or control (untreated) viruses were serially diluted and added to cells, which were incubated for 4 days. Viral titers were determined on day 4 post-infection by scoring each well for GFP-positive cells using a fluorescent microscope.

**Coagulation factor assays.** Control or laser-treated pooled normal plasma (George King Biomedical, Inc.) were thawed and analyzed using standard coagulation assays. Factor (F) II, FV, FVII, FVIII, FIX, FX, FXI, FXII, and fibrinogen were evaluated in Barnes-Jewish Hospital Laboratory, St. Louis, MO. Assays were completed on the IL ACL TOP 700 (Instrumentation Laboratories Company, Bedford, MA). Plasma levels of FII, FV, FVII, and FX were determined using functional assays based on the prothrombin time with human plasma immunodepleted of FII, FV, FVII, or FX. Similarly, plasma levels of FVIII, FIX, FXI, and FXII were determined using the activated partial thromboplastin time with human plasma immunodepleted of FVIII, FIX, FXI, and FXII. Instrumentation Laboratories Company reagents HemosIL RecombiPlasTin 2G and HemosIL SynthASil were used in the assay. Fibrinogen level was determined by a quantitative assay based on the Clauss method using Q.F.A. Thrombin (Bovine) reagent (Instrumentation Laboratories Company).

**Plasma protein gel electrophoresis.** For SDS-PAGE, control (untreated) or laser-treated samples containing equivalent quantities of plasma were boiled in loading buffer under reducing conditions and separated on a 10% gel. For native PAGE, control (untreated) or laser-treated
samples containing equivalent quantities of plasma were separated on a 10% gel under non-denaturing conditions. Protein bands were visualized with Coomassie blue staining (LabSafe Gel Blue, G-Biosciences).

**Fibrinogen protein preparation.** Purified human fibrinogen was obtained from Haemtech Technologies, Inc (Essex Junction, VT). For protein structure characterization measurements, fibrinogen solutions of 2.5 mg/mL were prepared by dissolving pure, lyophilized peptide in PBS buffer which was filtered with a 0.02µm Whatman Anotop25 filter. The actual concentration was checked by measuring the absorption of the sample with a Cary50 UV-Vis spectrophotometer (Agilent, Inc., Santa Clara, CA), using an extinction coefficient of fibrinogen at 280 nm of 5.12*10^5 M^{-1} cm^{-1}. Dynamic light scattering (DLS), circular dichroism (CD) and absorbance measurements were carried out immediately before and after irradiation of the sample.

**Dynamic light scattering measurements.** Fibrinogen samples were centrifuged at 12,000 × g for 15 min, and 5 µL aliquots were taken to measure the DLS signal of the sample immediately before and after irradiation. Autocorrelation functions of the scattered intensity at 90° scattering angle were collected at 25°C with a 5 sec acquisition time, using a Wyatt Technology DynaPro NanoStar with a 658 nm, 120 mW GaAs linearly polarized laser. Measurements of fibronogen were done in a 1 µL MicroCuvette (Wyatt Technology Corp., Santa Barbara, CA), previously calibrated with clear water. Data were analyzed using Wyatt Technology Dynamics 7 software,
using a regularization fit method to determine hydrodynamic radii of fibrinogen from the autocorrelation functions.

**Circular dichroism measurements.** Immediately before and after irradiation, fibrinogen samples were transferred to a 1 mm quartz cuvette (Starna) and circular dichroism spectra were measured at room temperature using a Jasco Instruments J-710 spectropolarimeter. Far UV spectra from 200 nm to 250 nm were obtained by averaging over eight scans, with a 1 nm bandwidth, 0.5 nm pitch and 50 nm/min scan speed, and then buffer subtracted. Fibrinogen concentration was measured from the absorption spectra, taken before and after laser irradiation, and used to convert CD signals to molar ellipticity. Slight variations in the signal amplitude were due to adsorption of fibrinogen on the walls of the vials.

**Results**

**USP laser treatment inactivates viruses in human plasma**

For this study, we chose HIV and HAV as medically significant enveloped and non-enveloped RNA viruses, respectively, and we chose MCMV as a representative enveloped DNA virus whose results could be extrapolated to relevant human pathogens such as cytomegalovirus and hepatitis B virus. To demonstrate that the USP laser treatment can inactivate viruses in plasma, aliquots of HIV, HAV, or MCMV were spiked into human plasma and treated with the
laser. USP laser treatment of virus-spiked plasma samples resulted in approximate 2-log, 1-log, and 3-log reductions in HIV, HAV, and MCMV titers, respectively (Figure 1A-C). The reduction in HAV titers after USP laser treatment exceeds that achieved by the currently licensed SD and amotosalen techniques\(^2\). It is anticipated that further optimization of laser parameters including laser power density, wavelength, and/or pulse width would yield greater inactivation of viruses. These data indicate that USP laser treatment can achieve clinically relevant reduction of viruses in human plasma.

Fig 1. Inactivation of viruses in plasma using a USP laser. Human plasma containing HIV (A), HAV (B), or MCMV (C) were treated with the USP laser. For the HIV-spiked plasma, viral titer was assessed by plaque assay in MAGI cells. For the HAV-spiked plasma, viral titer was assessed by plaque assay in fetal rhesus monkey kidney cells. For the MCMV-spiked plasma, viral titer was assessed by TCID\(_{50}\) assay in murine embryonic fibroblast cells. Results are shown as means ± SEM.
Table 1. Protein retention for USP laser-treated plasma. The function of individual coagulation factors in control (untreated) or USP laser-treated plasma was assessed using standard coagulation assays.

<table>
<thead>
<tr>
<th>Coagulation factor</th>
<th>Control plasma</th>
<th>Laser-irradiated plasma</th>
<th>Normal reference range</th>
<th>% retention after treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fibrinogen (mg/dl)</td>
<td>218 ± 4</td>
<td>158.5 ± 2.5</td>
<td>170 - 400</td>
<td>73</td>
</tr>
<tr>
<td>Factor II (IU/ml)</td>
<td>0.82 ± 0.02</td>
<td>0.98 ± 0.03</td>
<td>0.75 - 1.30</td>
<td>120</td>
</tr>
<tr>
<td>Factor V (IU/ml)</td>
<td>0.84 ± 0.01</td>
<td>0.80 ± 0.06</td>
<td>0.50 - 1.25</td>
<td>95</td>
</tr>
<tr>
<td>Factor VII (IU/ml)</td>
<td>0.92 ± 0.01</td>
<td>2.22 ± 0.23</td>
<td>0.50 - 1.75</td>
<td>241</td>
</tr>
<tr>
<td>Factor VIII (IU/ml)</td>
<td>0.72 ± 0.01</td>
<td>0.49 ± 0.01</td>
<td>0.50 - 1.60</td>
<td>68</td>
</tr>
<tr>
<td>Factor IX (IU/ml)</td>
<td>1.01 ± 0.01</td>
<td>0.96 ± 0.09</td>
<td>0.55 - 1.60</td>
<td>95</td>
</tr>
<tr>
<td>Factor X (IU/ml)</td>
<td>1.02 ± 0.03</td>
<td>1.04 ± 0.03</td>
<td>0.60 - 1.60</td>
<td>102</td>
</tr>
<tr>
<td>Factor XI (IU/ml)</td>
<td>1.01 ± 0.01</td>
<td>0.55 ± 0.09</td>
<td>0.60 - 1.40</td>
<td>54</td>
</tr>
<tr>
<td>Factor XII (IU/ml)</td>
<td>0.90 ± 0.00</td>
<td>0.69 ± 0.02</td>
<td>0.45 - 1.70</td>
<td>77</td>
</tr>
</tbody>
</table>

The function of human plasma proteins is preserved after USP laser treatment

To determine the functional integrity of human plasma after USP laser treatment, we laser-treated human plasma alone using the same conditions used to inactivate the viruses. After treatment, samples were subjected to standard coagulation assays. Human coagulation factors in laser-treated plasma showed preservation of activity comparable to that achieved with other clinically tested PR methods \(^2\). The percent retention of individual coagulation factors after laser treatment relative to control is shown in Table 1. Factors II, V, VII, IX, and X showed \( \geq 90\% \) retention. Factor XII and fibrinogen showed \( \geq 70\% \) retention. Factors VIII and XI were the most sensitive and showed 68\% and 54\% retention, respectively. The slight increase in Factor II activity (to 120\% control value) was not considered significant, as current PR methods have reported increases of similar magnitude in the activity of certain factors after treatment, probably due to intrinsic statistical variation in the assay \(^2\). Interestingly, the USP laser treatment caused a
dramatic enhancement in the measured activity of Factor VII in plasma (Table 1). This effect could either be direct (i.e., laser-induced structural changes in Factor VII protein) or indirect (i.e., laser-induced damage to factor(s) that inhibit Factor VII activity). We believe that the latter scenario is more likely, since we found that USP laser treatment of isolated Factor VII protein did not increase Factor VII activity as assessed by functional and chromogenic assays (data not shown). These results support a model in which the USP laser treatment may “inhibit an inhibitor” by damaging Factor VII inhibitor(s) that are present in human plasma (such as tissue factor pathway inhibitor [TFPI]), leading to an apparent “enhancement” in measured Factor VII activity. It is expected that further optimization of laser parameters including laser power density, wavelength, and/or pulse width would yield greater preservation of plasma proteins. These data demonstrate that USP laser irradiation retains coagulation factor activities in an acceptable range for clinical translation.
Fig 2. SDS-PAGE and native PAGE analysis of control and USP laser-treated plasma proteins. (A) On the left is shown the SDS-PAGE of control and laser-treated plasma; on the right, for comparison, is shown the SDS-PAGE of laser-treated MCMV virus adapted from Tsen et al. Control (untreated) or USP laser-treated plasma were boiled in reducing buffer and separated on a 10% gel. The solid arrow indicates the location of low mobility detergent-resistant aggregates; the dotted arrow indicates missing band(s) corresponding to aggregated proteins. (B) Native PAGE of control and laser-treated plasma. Control (untreated) or USP laser-treated plasma were separated on a 10% gel. Arrows indicate location of low mobility detergent-resistant aggregates.
USP laser treatment does not significantly generate detergent-resistant aggregates among human plasma proteins

Previous data supported a model whereby the USP laser inactivates viruses by laser-driven excitation of vibrational modes within viral capsids, resulting in aggregation of densely-packed tegument and capsid proteins. The laser treatment appeared to cause the formation of large, strongly bound aggregates of viral capsid/matrix proteins that did not readily dissociate under denaturing or reducing conditions, which we term “detergent-resistant aggregates.” This mechanistic explanation provides a potential basis of selectivity for laser inactivation of viruses in plasma whereby viral capsids, which exhibit low frequency vibrational modes that are excited by USP laser treatment, are damaged more severely by laser treatment while soluble proteins in plasma are relatively spared.

To determine the effects of laser treatment on human plasma proteins, we analyzed control (untreated) and laser-treated plasma by SDS-PAGE (Figure 2A). Both control and laser-treated plasma contained some intrinsic level of detergent-resistant aggregates as evidenced by the presence of low-mobility protein complexes unable to migrate through the gel. However, USP laser treatment of plasma did not cause any significant qualitative increase in these aggregates. A figure from a previous report, which shows dramatic USP laser-induced increase in detergent-resistant aggregates of MCMV viral proteins, is displayed alongside for comparison (Figure 2A). We were also unable to find evidence for any laser-induced increase in detergent-resistant aggregates in plasma by native PAGE (Figure 2B). These data suggest that
USP laser treatment does not induce the same type of detergent-resistant aggregation among plasma proteins as was seen for laser-treated MCMV proteins.

**Fig 3.** Structural analysis of control and laser-treated fibrinogen protein. Control (untreated) or laser-treated fibrinogen protein was analyzed by circular dichroism. The red line indicates the CD spectrum of control fibrinogen, while the dotted black line indicates laser-treated fibrinogen. The spectra show virtually complete overlap.

The secondary structure and aggregation state of purified human fibrinogen are unaltered after USP laser treatment.
As shown in Table 1, the function of individual coagulation factors was reduced after laser treatment. In particular, the function of fibrinogen, which is the coagulation factor that occurs at the highest concentration in plasma (~2–4 mg/ml) among the factors tested, was reduced to 73% of control. To determine if direct, laser-induced change in protein structure and/or aggregation state was responsible for this reduction in function, we analyzed the structure and aggregation state of purified human fibrinogen protein at physiological concentration (2.5 mg/ml) after USP laser treatment. We found no evidence for laser-induced alterations in the secondary structure of fibrinogen by circular dichroism (Figure 3). Furthermore, we did not observe any significant laser-induced aggregation of fibrinogen protein by dynamic light scattering, as evidenced by near-identical proportions of monomer in both control and laser-treated groups (Figure 4). We note that these values represent the percentage of scattered intensity from monomers (i.e. species with hydrodynamic radius of 12 nm). In both the controls and the irradiated samples the remaining 40% of intensity came from a much larger species, with a hydrodynamic radius around 100 nm. Because large aggregates scatter much more than monomers, these values indicate that roughly 90% of the protein mass was monomeric (assuming that the aggregates are spherical). Similar results were obtained with fibrinogen at lower concentrations (1.5 mg/ml and 0.6 mg/ml; data not shown). These findings indicate that USP laser treatment does not directly cause alterations in the secondary structure or aggregation state of fibrinogen protein when the protein is treated at physiological or sub-physiological concentrations.
Discussion

In this report we demonstrate broad-spectrum inactivation of viruses in plasma using a visible USP laser, with preservation of plasma proteins. Although we achieved clinically relevant reductions in virus, we note that the laser inactivation efficiency for viruses in plasma is decreased relative to that for viruses in phosphate-buffered saline (PBS)\textsuperscript{6,12}. This is likely due to the reduced penetration of light through plasma, which is less transparent than PBS. This can be remedied by designing a laser treatment chamber that is sufficiently thin (< 1 mm depth) to maximize light transmission. For example, in a continuous-flow treatment scenario, a tube or capillary with a small depth would ensure more complete exposure of the sample to the laser light.
Fig 4. Aggregation state of control and laser-treated fibrinogen protein. Control (untreated) or laser-treated fibrinogen protein was analyzed for aggregation state by dynamic light scattering. Values corresponding to the percentage of scattered intensity from fibrinogen monomers are shown.

After USP laser treatment, most coagulation factor activities were retained in the range of 70-100% control. Although these values are in the ballpark of other established PR methods, there is potential for improvement. Human plasma contains bilirubin, a molecule that absorbs light at the 425 nm wavelength we used for laser PR. This absorption leads to intermolecular energy transfers that may negatively affect the structure of bilirubin-associated proteins in
plasma. Since viruses in PBS alone are efficiently inactivated by USP laser treatment\textsuperscript{5-7,9-12}, bilirubin is clearly not required for laser PR. Thus it is possible that by selecting a different wavelength, i.e. 700nm where absorption by bilirubin is minimized, the detrimental effects of the USP laser treatment on plasma proteins can be mitigated.

We were unable to correlate the observed reduction in plasma protein function with any changes in the secondary structure or aggregation state of the proteins. There are two potential explanations for this. Firstly, the USP laser may cause only minimal structural damage to these proteins at a level undetectable by circular dichroism, dynamic light scattering, and SDS-PAGE. In this case, more sensitive methods such as nuclear magnetic resonance may be required to elucidate these changes. Alternatively, the function of plasma proteins might be indirectly inhibited by laser-induced damage to other factors in plasma that promote the coagulation activity of these proteins. Indeed, the absorption of laser light by other molecule(s) present in plasma, such as bilirubin, might lead to energy transfer from these molecule(s) to closely bound/closely associated proteins. Studies on the effect of bilirubin on plasma protein preservation after USP laser treatment are currently underway.

Visible USP laser treatment provides important advantages in the field of PR. The USP laser technology does not involve introducing potentially toxic or carcinogenic chemicals, and thus avoids possible side effects from such additives. Unlike the SD and amotosalen methods, which have seen significant clinical use in Europe, USP laser treatment can inactivate non-enveloped viruses. In addition, USP lasers are non-ionizing and do not disrupt covalent bonds, thereby reducing the concern of generating immunogenic neoantigens in blood products.
Furthermore, USP lasers are environmentally friendly, circumventing the need to incorporate undesirable compounds such as mercury which is used in UV lamps.

In conclusion, this is the first proof-of-concept study of PR of human plasma using USP laser technology. This chemical-free USP laser technology has potential advantages over current PR techniques. Application of this technology to other blood-borne viruses and optimization of the system design for large volume treatment are in progress.

**Acknowledgments**

We would like to thank Brenda Phelps for administrative assistance, Dr. Baogang Xu for assistance with reagents and assays, and Travis Chapa for assistance with viral assays. This work was supported in part by the Mallinckrodt Institute of Radiology Development Fund, NIH grants R01 EB008111, R33 CA123537 (SA), and NHLBI Ruth L. Kirschstein NRSA F30 grant HL116183-01 (SDT).

**References**


CHAPTER 6

A novel chemical-free inactivated whole influenza virus vaccine prepared by ultrashort pulsed laser treatment
Chapter 6 is a reformatted version of a submitted manuscript: “Shaw-Wei D. Tsen, Nisha Donthi, Victor La, et al. A novel chemical-free inactivated whole influenza virus vaccine prepared by ultrashort pulsed laser treatment (manuscript submitted)”. Under Dr. Samuel Achilefu’s supervision, my contributions to this work included designing and developing the ultrashort pulsed laser technology, carrying out the characterization and laser inactivation experiments, interpreting and analyzing data, and writing the manuscript. In this Chapter, I discuss my work in demonstrating a novel application for ultrashort pulsed laser technology toward the preparation of inactivated influenza vaccines.
Abstract

There is an urgent need for rapid methods to develop vaccines in response to emerging viral pathogens. Whole inactivated virus vaccines represent an ideal strategy for this purpose; however, a universal method for producing safe and immunogenic inactivated vaccines is lacking. Conventional pathogen inactivation methods such as formalin, heat, ultraviolet light, and gamma rays cause structural alterations in vaccines that lead to reduced neutralizing antibody specificity, and in some cases disastrous T helper type 2-mediated immune pathology. In this paper, we have evaluated the potential of a novel visible ultrashort pulsed laser method to generate safe and immunogenic whole inactivated virus vaccines without adjuvants. Specifically, we demonstrate that vaccination of mice with laser-inactivated H1N1 influenza virus at about 10-fold lower dose than that required using conventional formalin-inactivated influenza vaccines results in protection against lethal H1N1 challenge in mice. The virus, inactivated by the ultrashort pulsed laser irradiation, has been shown to retain its surface protein structure through hemagglutination assay. Unlike conventional inactivation methods, laser treatment did not generate carbonyl groups in protein, thereby reducing the risk of adverse vaccine-elicited T helper type 2 responses. Therefore, ultrashort pulsed laser treatment is an attractive potential strategy to generate whole inactivated virus vaccines with greater potency and safety than vaccines produced by current inactivation techniques.
Introduction

Emerging viral pathogens are a constant and prominent threat to human health worldwide, as evidenced by the recent outbreak of Severe Acute Respiratory Syndrome (SARS), Middle East Respiratory Syndrome (MERS) coronaviruses, and novel influenza strains with pandemic potential. In addition, there is a persistent risk of engineered viruses derived from bioterrorism. The most logical and cost-effective strategy to protect the human population from these emerging viral diseases is through immunization. Thus, there is a dire need for rapid methods to develop vaccines in response to new viral pathogens.

Whole inactivated virus (WIV) vaccines represent an ideal strategy for this purpose. In contrast to subunit or recombinant vector vaccines, WIV circumvents the need to identify relevant antigens and can be quickly produced from purified virus using chemical or physical inactivation methods. WIV contains many of the immunogenic epitopes and immunostimulatory components (such as toll-like receptor ligands) that are needed for an effective virus-specific immune response.

Unfortunately, a general method for producing safe and immunogenic WIV vaccines is lacking. Formalin, an alkylating agent, is one of the most extensively used methods for virus inactivation in the manufacture of vaccines. However, formalin treatment has several crucial limitations: (1) it causes structural damage to B-cell epitopes, which reduces the specificity of antibody responses elicited by the vaccine; (2) it generates carbonyl groups in proteins, a form of oxidative damage that induces harmful and undesirable T helper type 2 (Th2) -biased immune responses; and (3) it adds unnecessary cost and public concern by introducing a carcinogen into
the vaccine. These limitations can be seen in the failure of formalin-inactivated respiratory syncytial virus (RSV) and measles vaccines, which caused disastrous worsening of disease upon natural infection\textsuperscript{6-9}. Alternative methods for virus inactivation, including other alkylating agents, heat, ultraviolet (UV) light, and gamma radiation all suffer from various combinations of the abovementioned limitations\textsuperscript{10-13}.

Recently, a novel method for pathogen inactivation using visible ultrashort pulsed (USP) lasers has been reported (for a review, see Ref. 14). Visible USP laser treatment inactivates viruses through a physical mechanism: the impulsive stimulated Raman scattering (ISRS) process\textsuperscript{14,15}. There are several theoretical reasons why USP lasers would be advantageous over other methods for WIV vaccine production. Visible light (in the wavelength range of 400-700nm) lacks the photon energy to disrupt covalent bonds in biological macromolecules such as viruses; therefore, USP laser irradiation should not generate Th2 response-inducing carbonyl groups in proteins. In the absence of chromophores, visible light shows negligible intrinsic absorption by proteins and nucleic acids; thus, USP laser irradiation should not denature structural B-cell epitopes through heating. In addition, USP laser irradiation does not induce protein-nucleic acid crosslinking; hence, the TLR-stimulating capacity of viral nucleic acids should be preserved. Furthermore, the USP laser method does not involve introducing any potentially toxic or carcinogenic chemicals during treatment and would alleviate public concerns in those regards. These rationales make the USP laser treatment an attractive potential method to generate safe and effective WIV vaccines.
In this paper, we demonstrate that immunization of mice with the USP laser-inactivated whole inactivated H1N1 influenza virus at about 10-fold lower dose than that required by conventionally formalin- inactivated H1N1 vaccine results in protection against lethal-dose H1N1 influenza challenge. Splenocytes extracted from mice vaccinated with the USP laser-inactivated virus showed an enhanced influenza-specific cytotoxic T-cell (CTL) response compared to those from unvaccinated mice. In addition, we employed a neutralization assay to confirm the presence of neutralizing antibodies. We demonstrate through hemagglutination assay that the USP laser irradiation has no effect on the surface protein structure of the virus. Furthermore, we have evaluated the effects of USP laser-treatment on a model protein -- bovine serum albumin (BSA). We have found that, in contrast to conventional pathogen inactivation methods, the USP laser treatment did not generate Th2 response-inducing carbonyl groups in BSA protein. Therefore, ultrashort pulsed laser treatment is a novel and attractive potential strategy to generate WIV vaccines with greater potency and safety than vaccines produced by current inactivation techniques.

**Materials and Methods**

**Mice.** Female BALB/c mice (8 weeks old) were obtained from the National Cancer Institute (Bethesda, MD). The mice were kept in a pathogen-free environment at Johns Hopkins University (Baltimore, MD).
Cells. Madin-Darby Canine Kidney (MDCK) cells, obtained from ATCC (Manassas, VA), were used for all in-vitro assays. Cells were grown in Dulbecco’s modified Eagle’s Medium (DMEM) with L-glutamine and 10% FBS at 37°C with 5% CO₂.

Virus. The strain of virus used in this study was A/PR/8/34 (H1N1) from ATCC (Manassas, VA). The virus was first grown in MDCK host cells. After centrifugation at 1900 x g for 10 minutes at 4°C, the cell debris was removed. The remaining virus particles were then concentrated by centrifugation at 118,000xg for 1 hour at 4°C through a 20% sucrose cushion in phosphate buffered saline (PBS). The virus was stored in aliquots at -80°C. The titer of the virus was measured by the Tissue Culture Infectious Dose-50 (TCID₅₀) assay. For the TCID₅₀ assay, MDCK cells were plated on a 96-well plate. The virus was added in ten-fold dilutions using infection media (DMEM with 4 µg/mL N-acetylated trypsin and 0.03% BSA) for each successive row of wells. The plates were stored in an incubator at 37°C and 5% CO₂. After four days, formaldehyde was added to fix the cells and Naphthol Blue-Black was added to stain the fixed cells. The plates were washed and then the 50% infectious dose was calculated using the Reed-Muench method.

Laser Irradiation. The excitation laser source employed in this work was a diode-pumped mode-locked Ti-sapphire laser. The laser produced a continuous train of 65fs pulses at a
repetition rate of 80 MHz\textsuperscript{14,18,19}. The output of the second harmonic generation (SHG) of the Ti-sapphire laser was used to irradiate the sample. The excitation laser was chosen to operate at a wavelength of 415 nm and with an average power of 140 mW. It had a pulse width of full-width at half maximum (FWHM) of about 100 fs. An achromatic lens of focus length 7.5 cm was used to focus the laser beam into the sample area. In order to facilitate the interaction of the laser with the influenza virus particles, which were placed inside a Pyrex cuvette with buffer solution, a magnetic stirrer was set up so that virions would enter the laser-focused volume. All the laser-irradiated influenza samples contained approximately $2 \times 10^8$ TCID$_{50}$/ml virus and had a volume of about 200 μl. The typical exposure time of the sample to laser irradiation was about 8 hours. The diameter of laser beam was approximately 100 μm within the cuvette and the effective laser exposure time for individual virions was estimated to be about 28 seconds. The sterility of influenza virus samples after laser treatment was confirmed by TCID$_{50}$ assay. All the experimental results reported here were obtained at 23°C and with the single-laser-beam excitation. Temperature increase of sample solutions during USP laser treatments, as monitored by a thermocouple, did not exceed 2°C. The inactivated virus was stored in aliquots at -80°C for use in subsequent vaccination experiments.

**Hemagglutination assay.** Live and the USP laser-inactivated virus preparations were serially twofold-diluted in a 100 ml volume on a 96-well microtitre plate. A 0.5% chicken erythrocyte suspension was added to all wells and plates were incubated for 30 min on ice. This hemagglutination assay was adapted from Current Protocols in Microbiology\textsuperscript{20}. 

122
**Immunization and Challenge.** Groups of mice were vaccinated twice at a two week interval, as previously described $^{21,21}$, with $2 \times 10^8/\text{mL} \ \text{TCID}_{50}$ (20 μl) of laser-inactivated H1N1 virus by intranasal administration. Control mice did not receive vaccine. The mice in both groups maintained a consistent weight prior to administration of the challenge dose. Two weeks after the last vaccination, the mice were challenged with a lethal dose ($6 \times 10^2 \ \text{TCID}_{50}$) of H1N1, and the weight of the mice was monitored daily. Mice losing more than 25% of their body weight were considered to have reached the experimental endpoint and were euthanized.

**Flow cytometry.** Two weeks after the last vaccination, mice were sacrificed and their splenocytes were isolated and stimulated using influenza nucleoprotein peptide (NP). Detection of cellular surface CD8a and intracellular IFN-$\gamma$ was performed using flow cytometry as described previously$^{23}$. Briefly, the cells were incubated for overnight with 1 μg/ml of GolgiPlug (BD Pharmingen) in the presence of 2 μg/ml of NP peptide. After washing with FACScan buffer, the cells were stained with phycoerythrin-conjugated anti-mouse CD8a antibody. The cells were then incubated with BD cytofix/cytoperm solution (BD Pharmingen) followed by staining with FITC-conjugated anti-mouse IFN-$\gamma$ antibody. The splenocytes of all the mice in each group were pooled together and then analyzed by flow cytometry on a Becton-Dickinson FACSCalibur with CellQuest software (BD Biosciences, Mountain View, CA). Gating was performed on the lymphocyte area.
**Microneutralization Assay.** Blood was collected from the tail vein of vaccinated (n=5) or unvaccinated (n=4) mice two weeks after the last vaccination. The serum was stored in aliquots at 4°C. After the serum was collected, the mice were tested for H1N1-specific neutralizing antibodies as follows. 2x10^4 MDCK cells were plated in each well of a 96-well plate. Serum was diluted with infection media (DMEM with 4 µg/mL N-acetylated trypsin and 0.03% BSA) to 1:100 and added to the first row of wells, containing the MDCK cells. After thorough mixing of the well contents, 25 µl of the first row’s wells were added to 25 µl of infection media in the next row. This procedure was continued until the last row of wells resulting in two-fold dilutions and the extra serum was discarded. A constant H1N1 concentration of 1.75x10^5 TCID₅₀/well was used for each plate. The virus and serum were incubated at 25°C for two hours and then added to the 96-well plate with MDCK cells. The plates were stored for three nights in an incubator at 37°C and 5% CO₂. Formaldehyde and Naphthol Blue-Black was added to visualize the results of the reaction as in the TCID₅₀ assay. This assay was repeated three times. Neutralization titers were calculated using the Reed-Muench Method. The inverse of the highest dilution at which 50% protection was achieved was determined to be the neutralization titer of the serum²⁴.

**ELISA.** The levels of anti-influenza antibodies in sera were determined by a direct enzyme-linked immunosorbent assay (ELISA) as described previously²⁵,²⁶. Briefly, wells of a 96-microwell plate were coated with 100 µl of a 4 µg/ml of influenza and incubated at 4°C overnight. The wells were then blocked with PBS containing 20% fetal bovine serum. Sera were prepared from the mice on day 14 post-immunization, 100 times diluted in PBS, added to the
ELISA wells, and incubated at 37°C for 2 hours. After being washed with PBS containing 0.05% Tween 20, the plate was incubated with a 1/2,000 dilution of a peroxidase-conjugated rabbit anti-mouse immunoglobulin G antibody (Zymed, San Francisco, CA) at room temperature for 1 hour. The plate was washed six times, and then 1-Step Turbo TMB-ELISA was used as a substrate for color development (Pierce, Rockford, IL); color development was stopped with 1 M H₂SO₄. The absorbance of the ELISA plate wells was then determined with a standard ELISA reader at 450 nm.

**Protein carbonyl quantification.** The carbonyl concentrations in untreated BSA, USP laser-treated BSA, or UV-treated BSA were assessed using a dinitrophenylhydrazine (DNPH)-based protein carbonyl colorimetric assay kit (Cayman Chemical) according to the manufacturer’s instructions. In the UV treatment group, BSA samples were exposed to a Phillips TUV30W 30-Watt germicidal UVC lamp at a 4 cm distance from the bulb for 90 min.

**Statistical Analysis.** Data was analyzed using Microsoft Excel 2010. Each experiment was repeated in duplicate or triplicate. Individual data points were compared using Student’s t-test.
Results

Effect of the USP laser virus inactivation on hemagglutination activity

Hemagglutination activity after the USP laser virus inactivation provides one indicator as to the structural alternation of the surface proteins of the virus inactivation treatment. Purified influenza stock was aliquoted into batches and treated with the USP laser irradiation. Following the complete loss of infectivity, we compared the hemagglutination activity of live and inactivated viruses. As shown in Table 1, within the experimental uncertainty, hemagglutination activity was not affected by the USP laser irradiation. These results provide evidence that the USP laser irradiation, among other inactivation methods, causes the least structural changes of viral surface proteins.

Table 1. Hemagglutination activity of live and the USP laser-inactivated influenza virus.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Method of inactivation</th>
<th>HAU/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>A/PR/8/34</td>
<td>Original live stock</td>
<td>$(2.3 \pm 0.2) \times 10^5$</td>
</tr>
<tr>
<td></td>
<td>USP laser inactivation</td>
<td>$(2.1 \pm 0.2) \times 10^5$</td>
</tr>
</tbody>
</table>
Laser-inactivated H1N1 influenza vaccine confers protection against lethal H1N1 challenge in mice

We determined whether the laser-inactivated H1N1 virus vaccine conferred protection against a lethal dose of H1N1. Percent change in initial weight was used as an indicator of the health of the mice. Groups of mice received vaccination (n=8) or no treatment (n=8). Two weeks after the last vaccination, mice in both groups were given a lethal dose of $6 \times 10^2$ TCID$_{50}$/mL live H1N1 virus. Following administration of the challenge dose, the weights of mice in the control group decreased rapidly. As shown in Figure 1, by the seventh day after receiving the challenge dose, control mice lost a significant percentage of their initial weight while the mice in the vaccinated group maintained healthy weights. Our results show that vaccination is 87.5% effective against a challenge of lethal dose.
Fig 1. Body weight changes in H1N1-challenged mice. Groups of BALB/c mice (n=8) were vaccinated twice with $2 \times 10^8$ TCID$_{50}$ (20 μl) of laser-inactivated H1N1 virus by intranasal administration at a two week interval, or received no treatment. 21 days later, all mice were challenged with a lethal dose of $6 \times 10^2$ TCID$_{50}$/mL of A/PR/8/34 influenza virus. Following lethal challenge, mice were monitored for percent change of initial weight for 9 days. Each point represents the mean and bars represent standard deviation. (Student’s t test comparing the final weights of each group of mice, $P=0.026$).

Laser-inactivated H1N1 influenza vaccine increases CD8+ immune response in mice

To further investigate the immunity generated by vaccination with laser-inactivated virus, CTL activation was assessed. Splenocytes were obtained from vaccinated or untreated mice and stimulated by immunogenic influenza NP peptide. Cells were fluorescently tagged for CD8 and IFN-γ and analyzed by flow cytometry. Figure 2 demonstrates that the vaccinated mice show a 10-fold increase in the percentage of activated NP peptide-specific T cells in comparison to the
control mice. These data indicate that vaccination with laser-inactivated virus generates influenza antigen-specific CD8+ T cell immune responses.

**Fig 2.** CD8+ T cell induction following vaccination. Splenocytes were isolated from vaccinated and untreated BALB/c mice and then the cells were incubated overnight in the presence of 2 μg/ml of NP peptide. After washing with FACScan buffer, the cells were stained with phycoerythrin-conjugated anti-mouse CD8a antibody, followed by fixing/permeabilizing and staining with FITC-conjugated anti-mouse IFN-γ antibody. The splenocytes were then analyzed by flow cytometry on a FACSCalibur with CellQuest software, and gated on the lymphocyte area. a) Representative flow cytometry analysis. The upper right-hand quadrant shows the percentage of NP-specific, IFN-γ secreting CD8+ T cells among lymphocytes. b) Bar graph depicting the percentage of activated CD8+ T cells among splenocytes in vaccinated and unvaccinated mice (P < 0.001).

**Laser-inactivated H1N1 influenza vaccine generates influenza-specific neutralizing antibodies**

We next investigated humoral immunity induced by vaccination using a microneutralization assay. This neutralization assay is a sensitive and specific technique to measure neutralizing antibody responses to H1N1 virus. As shown in Figure 3, we found that
sera from mice that received laser-inactivated vaccination showed a significantly higher neutralizing antibody titer compared to sera from control (unvaccinated) mice. Furthermore, we observed that vaccination with decreasing doses of laser-inactivated influenza virus generated virus-specific antibody responses to lesser degrees (results not shown). These experimental results indicate that vaccination with laser-inactivated virus induces neutralizing antibody immune responses.

Fig 3. Neutralizing antibodies detected by microneutralization assay. Serum from vaccinated (n=5) and unvaccinated (n=4) groups of mice was extracted 20 days after vaccination and then added to MDCK cells in a 96-well plate, serially diluted, and incubated for three days. Subsequently, a constant H1N1 concentration of $1.75 \times 10^5$ TCID$_{50}$/well was used for each plate. The virus and serum were incubated at 25°C for two hours and then added to the 96-well plate with MDCK cells. The plates were stored for three nights in an incubator at 37°C and 5% CO$_2$. Formaldehyde and Naphthol Blue-Black was added to visualize the results of the reaction. The presence of neutralizing antibodies was determined by cell survival. Neutralization titers were calculated using the Reed-Muench Method.
TEM images reveal that the laser-inactivated virus retains its global viral structure

We used transmission electron microscopy (TEM) to visually examine the active and laser-inactivated influenza viruses. Figures 4A and 4B show the TEM images of the influenza virus with or without laser irradiation, respectively. We have found that the global structure of the virus is maintained after the USP laser treatment, indicative of the production of WIV by the USP laser irradiation.

**Fig 4.** Transmission electron microscopy images of influenza virus. Samples of laser-inactivated and active A/PR/8/34 influenza virus were fixed with glutaraldehyde and visualized using transmission electron microscopy. (A) Fixed sample of laser-inactivated virus at 93000x magnification. (B) Fixed sample of the active virus at 93000x magnification. These TEM images demonstrate the retention of capsid and global structure of the influenza virus after the
USP laser irradiation and provide support for the laser-induced protein aggregation through ISRS process for the most likely inactivation mechanism.

**Fig 5.** Carbonyl content of laser-treated BSA protein. The carbonyl concentrations in untreated BSA, USP laser-treated BSA, or UV-treated BSA were assessed using a DNPH protein carbonyl colorimetric assay kit. Untreated BSA served as negative control; UV-treated BSA served as positive control.

Laser treatment does not generate carbonyl groups in protein

Conventional inactivation methods including formalin, UV treatment, and gamma radiation are potent inducers of carbonyl groups in protein\(^4,10,11,27\); these carbonyl groups are in turn inducers of harmful and undesirable T helper type 2 responses \(^4,5\). To determine whether
laser treatment generates protein carbonylation, we used a colorimetric 2,4-dinitrophenylhydrazine (DNPH)-based assay to quantitate carbonyl content in laser-treated BSA. Untreated BSA or UV-treated BSA served as negative and positive controls, respectively. Figure 5 shows that there is no significant increase in carbonyl content in laser-treated BSA samples relative to untreated BSA. By contrast, the carbonyl content of the UV-treated BSA was dramatically increased relative to both untreated and laser-treated BSA (p=0.0004). These results demonstrate that USP laser treatment generates fewer carbonyl groups in protein antigens compared to conventional pathogen inactivation methods, thus reducing the risk of detrimental vaccine-elicited Th2 responses.

Discussion

It has been shown that non-enveloped viruses such as murine norovirus (MNV) are inactivated by a visible USP laser through the ISRS process\textsuperscript{15}, whereby the USP laser pulses excite Raman-active vibrational modes on the capsid of the non-enveloped virus -- MNV. The amplitude of the vibration is linearly proportional to the laser intensity. When the laser intensity is sufficiently large, the excited amplitude of vibration on the capsid can become extremely large, leading to the breaking of weak links such as hydrogen bonds and hydrophobic contacts in the capsid of the virus. This causes the capsid to disintegrate into subunits. The virus becomes inactivated because of the loss of integrity of its capsid. On the other hand, it has been demonstrated that enveloped viruses such as murine cytomegalovirus (MCMV) are inactivated by a visible USP laser via the ISRS process as well but through a different route/pathway\textsuperscript{19}. The
USP laser pulses excite Raman-active modes on the proteins of the capsid as well as the tegument proteins of the enveloped virus -- MCMV through the impulsive stimulated Raman scattering process. The excitations, which break the hydrogen bonds and hydrophobic contacts on these proteins, cause partial unfolding of the proteins. These unfolded proteins will rapidly reform their broken weak hydrogen bonds and hydrophobic contacts and return to their original folded configuration. However, if the concentration of proteins is very high, such as in the case of proteins confined within the capsid of a virus, they can form hydrogen bonds and hydrophobic contacts with other proteins nearby, leading to the aggregation of proteins. This has been shown to be the case for the enveloped virus – MCMV. Aggregation between capsid proteins and tegument proteins has been found to be the cause of inactivation for an enveloped virus. All influenza viruses are enveloped viruses. Therefore, we believe that the most likely inactivation mechanism for the H1NI influenza virus inactivated by the visible USP laser studied here is the ISRS process. The TEM images of H1N1 influenza virus before and after the USP laser irradiation, shown in Figure 4, further support our argument. The striking similarity between these TEM images to those of MCMV \cite{19}, i.e., the retention of the capsid and the global structure of the virus, suggests that the effects (and therefore the underlining inactivation mechanism) of the USP laser irradiation on these two enveloped viruses are the same.

To give a perspective on the vaccine potency reported in our current study, it is illustrative to compare the vaccine dosages required for protection against lethal challenge in mice using conventionally- or laser-inactivated H1N1 influenza vaccines. Among conventional methods including heat, formalin, beta propiolactone, and detergents, formalin was found to have the greatest preservation of influenza vaccine antigens \cite{13}. A formalin-inactivated H1N1 vaccine
required two doses at \( \sim 1.6 \times 10^7 \) pfu/dose for 60% protection\(^{28}\). With the visible USP laser irradiation method, we employed two doses at \( \sim 2.76 \times 10^6 \) pfu/dose, which is a nearly 10-fold lower dose relative to the formalin vaccine, to achieve 87.5% protection. These results indicate that the USP laser-inactivated vaccine is significantly more efficient than the vaccine prepared by formalin.

The relatively high potency of the USP laser-inactivated influenza virus vaccine can be partly attributed to the fact that the visible USP laser irradiation has minimal effects on the structure of proteins. The circular dichroism (CD) spectrum of bovine serum albumin (BSA) protein measured before and after the USP laser irradiation is a good example. The CD spectrum is very sensitive to the secondary structure of proteins. It has been shown that, within experimental uncertainty, there is no change in the CD spectrum of BSA protein before and after USP laser irradiation\(^{14}\). These spectroscopic results are in line with our hemagglutination activity results. Our experimental results on the hemagglutination activity of the virus shows that, within the experimental uncertainty, the USP laser irradiation has no effects on the surface protein structure of the virus; whereas formalin inactivation resulted in a ninefold reduction in hemagglutination titres\(^{28}\).

We note that the USP laser-inactivated influenza vaccine may generate heterosubtypic immunity, which is the aim of current efforts to design universal influenza vaccines. The CTL response is a key mechanism for improved heterosubtypic protection against influenza because CTLs have been shown to be specific for epitopes that are conserved among viral subtypes\(^{29}\). Our data showed that splenocytes from vaccinated mice showed a ten-fold increase of influenza
NP-specific CTLs compared to unvaccinated mice. These results suggest that the laser-inactivated influenza vaccine has the potential to generate cross-protection against multiple strains and thus address the issue of viral mutation.

The presence of carbonyl groups in vaccine antigens has been linked to the induction of undesirable and potentially deleterious Th2-mediated immunopathology\textsuperscript{4,5}. Many conventional inactivation techniques including formalin, UV, and gamma radiation are potent inducers of protein carbonylation\textsuperscript{4,10,11,27}. In contrast to these techniques, visible USP lasers lack the energy to disrupt covalent structures in proteins. Therefore, we reasoned that USP laser treatment would not cause protein carbonylation. The experimental results in Figure 5 confirm this. These data demonstrate that USP laser treatment does not generate significant levels of carbonyl groups in protein antigens compared to conventional pathogen inactivation methods, thus reducing the risk of detrimental vaccine-elicited Th2 responses. In addition to carbonyl groups, other types of covalent damage caused by formalin, UV, and gamma radiation can result in the formation of “neoantigens” and elicit adverse immune reactions when administered to patients, as was seen in penicillin allergies\textsuperscript{30} and certain chemically-treated blood products\textsuperscript{31}. In contrast, the USP laser inactivates enveloped influenza virus through the disruption of weak, non-covalent hydrogen bonds and hydrophobic contacts in the virion, leading to the aggregation of capsid proteins and tegument proteins. As a result, there is an overall reduced concern of side effects from vaccines prepared by the USP laser treatment method.

In summary, we have demonstrated a novel USP laser irradiation method for the production of safe and potent whole inactivated virus vaccines. We envision that the future of
Pathogen inactivation technologies will favor chemical-free methods that target properties specific to pathogens while preserving desirable components of the treated product, leading to improved safety profiles. The USP laser irradiation method we have presented in this report is one such potential technology. Further evaluation of the USP laser-inactivated vaccine for cross-protection and in the context of other important pathogens such as HIV, SARS, and MERS is warranted.

Acknowledgments

This work was supported in part by NHLBI Ruth L. Kirschstein NRSA F30 grant HL116183-01 (SDT), the Mallinckrodt Institute of Radiology Development Fund, and NIH grants R01 EB008111 and R33 CA123537 (SA).

References


CHAPTER 7

Conclusions
Emerging pathogens rank highly on the list of potentially catastrophic events that can impact humans. Historical examples of emerging pathogens include hepatitis C virus and human immunodeficiency virus (HIV), which caused thousands of individuals to become infected through blood transfusions in the 1970s and 80s. More recently, we have been confronted with airborne threats such as Severe Acute Respiratory Syndrome (SARS) virus, novel strains of influenza, and Middle East Respiratory Syndrome (MERS) virus. Therefore, a technology is needed to inactivate pathogens which would allow the production of safe blood products for transfusion, and the preparation of safe and effective inactivated vaccines.

Unfortunately, the existing pathogen inactivation techniques do not adequately meet this need. Chemical methods such as detergents, photosensitizers, and formalin introduce foreign compounds into the product, which are impossible to completely remove and often pose significant risk of side effects. Physical methods such as heat, ultraviolet (UV) light, and gamma radiation all cause extensive denaturation and/or changes to the covalent structure of proteins. These effects reduce the safety and efficacy of the treated product.

In these regards, ultrashort pulsed (USP) lasers represent a potentially ideal technology for pathogen inactivation. We have demonstrated the capability of visible USP lasers to inactivate a broad range of viruses including HIV, influenza virus, hepatitis A virus, human papillomavirus (HPV), and murine cytomegalovirus (MCMV), while preserving mammalian proteins and cells. We have revealed substantial evidence that the USP laser inactivates viruses by damaging viral capsids through impulsive stimulated Raman scattering, a total paradigm shift from conventional techniques. We have developed a model for USP laser-mediated virus
inactivation whereby laser-mediated aggregation of capsid proteins leads to stabilization of viral capsids, resulting in defective intracellular capsid disassembly and loss of viral infectivity. Furthermore, we have demonstrated that the USP laser technology can be used for pathogen reduction of human plasma, and for generating safe and potentially more effective influenza virus vaccines.

In future research we will expand our mechanistic studies to a broader range of viruses to confirm the universality of our inactivation model. We will further optimize the USP laser treatment parameters to achieve clinically relevant reductions in viruses while preserving the function of human plasma, and extend these applications toward red blood cells and platelets. We will test whether the USP laser-inactivated influenza vaccine shows an enhanced safety and efficacy profile relative to conventionally inactivated influenza vaccines. Furthermore, we will investigate the use of the USP laser in the preparation of vaccines for emerging viruses and viruses for which no vaccine currently exists. From discovery to bench studies to real-world applications, this novel USP laser technology represents a unique and promising pathogen inactivation method to address the issue of emerging pathogens.
List of Graduate Publications


