Electrophoresis as a technique for direct analysis of laser-tissue ablation products

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ABSTRACT

Polyacrylamide gel electrophoresis is a technique that allows the constituents of complex biological mixtures to be resolved with respect to their molecular weight. This is a technique that has been used to identify protein or nucleic acid fragments, and it is now being applied to allow molecular analysis of laser-tissue ablation products. The hope is to allow for a greater understanding of the ablation process than is currently possible, as the effects of the interaction at a molecular level can be identified directly rather than inferred from other related phenomena such as mechanical, acoustic or thermal measurements. Here the preliminary work using this technique is presented, and plans for further work in the autumn at FELIX are outlined.

Keywords: Ablation, Analysis, Infrared, Electrophoresis, Molecular weight

1. INTRODUCTION

There have been many studies examining infrared ablation of soft tissues in the past twelve to fifteen years. These have looked at ablation efficiency, collateral damage, effects of pulse structure, wavelength and power on efficiency, and practical issues such as delivery of the beam. Whilst all of these studies are useful in examining the manifest effects of the interaction, there has been little progress in examining the fundamental mechanisms of the ablation. This is an area that will be covered later in this introduction, but a broad introduction to the merits of various wavelength regimes follows first.

1.1 Wavelengths and their effects

The infrared region of the spectrum is attractive for medical ablation of soft tissues for several reasons. Mainly, infrared (IR) radiation lacks the carcinogenic potential that has been shown to be a part of ultraviolet (UV) laser tissue interaction1-7. UV is not only a potential problem for patients undergoing surgery, but could also be a long-term risk to the surgeons and other medical staff using the systems on a routine basis. Additionally, existing mid-infrared laser systems (0.7 – 3 µm) tend to be solid state lasers, and are thus compact, less demanding in terms of maintenance and servicing than UV systems, and are normally cheaper than UV gas lasers8.

Thus far there has been no mention of the drawbacks of conventional IR laser systems. These drawbacks include a tendency to cut less cleanly than UV lasers, and to have a larger zone of thermal and mechanical damage surrounding the incision site than a comparable UV system. Indeed early IR lasers exhibited extremely ragged cutting edges when compared to their UV counterparts8, 9. It is also notable that currently the most promising wavelengths in the IR are not accessible with practical sources, but this could potentially change with the advent of Quantum Cascade (QC) lasers10-12.

Before going any further into the analysis of the ablation process, it is perhaps useful to have a summary of the ablation

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mechanisms as they are currently understood.

1.2 Simple IR Ablation Mechanism

Traditional pulsed laser ablation is normally achieved through a combination of photothermal and photomechanical effects. Thermal denaturation weakens the structural matrix of the tissue, while explosive transition of tissue water to high-pressure vapor then ruptures the structural matrix, propelling the ablated material from the site of irradiation. In situations where there is a tissue-air boundary, this second mechanism is clearly seen as an ablation plume; this has been documented in various practical and theoretical studies. It is generally accepted that there is a trade off between thermal damage to surrounding tissue caused by longer pulses (generally >100ms), and mechanical damage caused by short pulses (<1ms). Explosive transition ablation produces a region of heat-affected tissue, as its mechanism is entirely thermal. A significant area surrounds the site of ablation where heating was insufficient to remove tissue, but where the temperature rise was sufficient to cause damage, and several studies have examined the thermal behavior of the system during ablation.

2. CURRENT METHODS FOR ABLATION ANALYSIS

2.1 Ultrasonics

Ultrasonic detection has been used by a variety of authors as a method of determining the ablation efficiency of a laser system. Uhlhorn et al supposed that the acoustic energy resulting from an ablation process was an indicator of the strength of the ablative event. The acoustic energy from materials where water is the primary chromophore would be expected to increase with water content. By comparing wavelengths where there was only water coupling with those where there was also coupling into the Amide II Protein bands, Uhlhorn showed a statistically significant difference between the two regimes.

Paltauf et al showed that “the mechanical action of thermoelastic stress waves is characterized by high stress amplitudes but low energetic efficiency. A model combining spallation and vaporization is therefore proposed for efficient tissue ablation.” This is significant, as this suggests a more complex model than that traditional photothermal mechanisms, suggesting that in fact a combination of photothermal and photomechanical effects may be responsible for determining ablation performance.

2.2 Stress wave detection

Dingus and Scammon introduce an interesting theoretical discussion of the importance of Grüneisen-stress effects in ablation. He describes how the ablated tissue is removed by surface spallation, where heating of the front surface causes a positive compressive stress in the region, whose amplitude can be calculated by the Grüneisen coefficient. The tissue will dynamically expand both forwards and back into the tissue, and the pulse will be reflected at the front surface, producing a negative (tensile) reflected pulse. This tensile pulse may well exceed the tensile strength of the tissue, and a section is thus ablated from the surface of the tissue if the interface is with a gas or vacuum. This behavior was demonstrated practically by Itzkan in 1994.

Tribble and Uhlhorn have both used this phenomenon to monitor the ablation process. Tribble et al demonstrated that the stress transients at 3 μm had a sole early process during the ablation pulse, whilst there was an additional slower process at 3.36 and 6.45 μm. Uhlhorn et al used the technique to demonstrate that the thermomechanics of the ablation process at all Free Electron Laser (FEL) generated IR wavelengths, when applied to gelatin and soft tissue, more closely resemble that of a UV laser than IR ablation. This may indicate that the unusual pulse structure of the FEL may be highly significant in the ablation behavior of the system.

2.3 Ablative recoil studies

A similar but slightly different technique was used by Auerhammer et al to evaluate the ablative recoil momentum of their system. Here a small 2 x 2 mm section of swine cornea was attached to a “sensitive, almost massless” pendulum. When ablated, the movement of the pendulum was in direct response to the ejection of the ablation plume, thus
allowing a numerical calculation of the recoil momentum, and thus the induced kinetic energy introduced into the
system.

2.4 Laser flash photography

Lasers can be used to allow high-speed photography of the ablation plume, and to allow for visualization of bubble
formation in a plane. Using this technique, the dynamics of the plume formation can be examined and quantified, and
criteria such as ejection velocity, particle size and plume shape can be identified. All of these give clues as to the action
of the ablation mechanism. For example, Auerhammer’s group showed that whilst the ablation performance, by some
criteria, of 6.2 and 6.45 \( \mu \text{m} \) interactions is almost identical, the 6.45 \( \mu \text{m} \) radiation produces a finer cloud of particles
than the 6.2 \( \mu \text{m} \)\(^{16}\).

Tribble’s team used laser photography to monitor the size of the ablation plume by casting a continuous wave HeNe
laser sheet over the site of irradiation\(^{14}\). By monitoring the strength of the collected signal, the density of the plume
could be monitored as a time-resolved measurement. This showed clear differences in the dynamics of various sources,
as discussed above.

Pulsed laser sheets can also be used to allow for high speed visualization of the bubble dynamics of laser ablation of
fluid\(^{24}\). Here visualization of the unexpected bubble formations due to the interaction of Er:YAG laser light (2.94 \( \mu \text{m} \))
with water vapor formed during the ablation process was clearly highlighted, with the formation of a lobed bubble
showing the effects of vapor formation on the interaction.

2.5 Histological examination of tissue cross section

Edwards et al showed the potential of 6.45 \( \mu \text{m} \) radiation to give good cutting results largely by histological analysis of
tissue samples\(^{25}\). He was able to show cross sections of tissue that had been ablated by radiation at 3.00 and 6.45 \( \mu \text{m} \),
clearly showing the additional collateral damage inflicted at 3 \( \mu \text{m} \).

A less conventional method for analyzing the ablation crater was shown by Bachman et al, who used silicon casts to
show the corneal topography after ablation\(^{26}\). To quote their paper, “A three-dimensional cast of cornea after irradiation
was made by using a two-component silicon gel that polymerizes within minutes, thus preserving the corneal
topography immediately after photoablation.” They listed the advantages of this technique as including it being “...non-
destructive, accurate, inexpensive, practical, and reduces requirements for laboratory animals.”

2.6 IR Laser Performance Improvements

In the last ten to fifteen years much progress has been made in improving the performance of IR lasers. A variety of
wavelengths have been tested using various laser sources and with varying results. Water forms the primary IR
chromophore in most biological tissues\(^{16}\), and dynamic differences in the behavior of laser systems are mainly
influenced by the water absorption at that wavelength. A summary of the absorption coefficients of water and collagen
prepared by Peavey et al\(^{27}\) clearly shows the variation in absorption across the mid infrared region, and also
interestingly highlights the potential for chromophores other than water at other specific wavelengths. For example,
Jean and Bende showed that ablation efficiency and behavior vary widely when tuning between 2.7 and 6.7 \( \mu \text{m} \) using a
FEL\(^{28}\), and later studies by Auerhammer et al showed a distinct variation in plume density and behavior when tuning
between 6.1 and 17.5 \( \mu \text{m} \)\(^{16}\).

Varying the pulse length and shape to give better ablation characteristics has had some success in more conventional
laser systems operating at fixed wavelengths. Pulse length will, along with the optical properties of the tissue, determine
whether the laser energy will be unconfined, thermally confined or stress confined\(^{8}\). In a thermally confined system
distribution of thermal energy within the tissue will be entirely determined by the laser light distribution. This is
because the laser pulse duration is shorter than the thermal diffusion time of the tissue, and therefore all thermal energy
is deposited within the spot. If sufficient energy is deposited to allow photo vaporization to take place, this will result in
the ejection of tissue.
In a stress confined system the laser pulse length is shorter than the period of time that the shock wave takes to travel out of the irradiated volume. This situation can lead to extremely large shockwaves being generated that can cause substantial damage to surrounding tissues. However, evidence is emerging that sufficiently short pulses in the picosecond regime may allow for extremely precise cutting.

3. GAPS IN CURRENT ANALYSIS TECHNIQUES AND A POTENTIAL SOLUTION

All the analysis techniques described above share one common fault. They all allow for analysis of the final ablation results, or allow an inference of the mechanism, but none directly illuminate the actual ablation mechanism. This is a serious failure, as whilst much information can be gleaned from the various methods, it is still not possible to state categorically what physical parameters are changing that directly influence the ablation dynamics.

Much has been speculated about the ablation chemistry, but no studies have been made in any detail. What would be interesting would be to analyze the molecular effects of the laser tissue interaction. In particular, for pulses of equal intensity it would be interesting to study the degree of molecular damage directly induced by the laser energy, as it is this factor that would primarily influence the ablation dynamics if the thermal model of ejection is correct. Up to this point this direct analysis of the molecular effects has not been possible, and it is this point that this research is aiming to address.

Electrophoresis is a technique long used in biology. It has been used to identify protein or nucleic acid fragments, and it is now being applied for the first time (as far as we know) to allow molecular analysis of laser-tissue ablation products. The work is still very much under development, and final results are not available in time for this paper, however the general technique is described, along with its method of application, and its potential to aid understanding of the ablation process outlined.

4. METHODS

For the development work, gelatin was used as an optical tissue phantom that closely mimics the optical behaviour of typical biological tissues. By varying the concentration of gelatin by volume compared to the water then various protein concentrations can be obtained allowing for simulation of the major biological tissues.

4.1 Gelatin preparation

Food grade gelatin (Supercook) was used in the experiments. Gelatin concentrations of 3, 5, 10 and 20% gelatin by weight in distilled water were prepared. The molten gelatin was poured into shallow trays to a depth of approximately 2.6 mm and allowed to set at room temperature.

4.2 Collagenase preparation

Collagenase (Sigma Crude Collagenase, General Use, Type 1A) was made up to concentrations of 1, 0.5, 0.25, 0.125, 0.0625, 0.03125 and 0.0156 mg/ml by serial dilution.

4.3 Ablation simulation

In the absence of a suitable high-powered laser source at Warwick, collagenase is used to cause molecular fragmentation in order to simulate the ablation of the gelatin and to allow for development of the analysis technique. Collagenase at 1, 0.5, 0.25, 0.125, 0.0625, 0.03125 and 0.0156 mg/ml was prepared and placed on the gelatin at pre-marked spots on the tray, 5 ml of collagenase per spot, with two spots for each concentration of collagenase per gel. The gels were then incubated at room temperature overnight sealed in plastic bag to avoid evaporation.

4.4 Protein Sampling

Digested protein is sampled by absorbing a small section of tissue onto a small, 1.5 x 1.5 mm piece of filter paper. This paper is then placed in 50 ml of sample buffer (non-reducing sample buffer prepared in-house, 0.125M tris/HCl, pH6.8; 4%SDS; 20% glycerol and 2 mg bromophenol blue) along with a protease inhibitor (Sigma Protease Inhibitor Cocktail,
General Use) in an eppendorf tube. This inhibitor stops any further degradation of the proteins by enzymic action, whether collagenase induced or as a natural process in tissue, which could influence the results of the test.

The sample buffer and filter paper are boiled for ten minutes in order to maximize the protein recovered. The eppendorf is then micro centrifuged at 13,000g for one minute to separate the filter paper from the buffer. A small (5 µl) sample is then removed, and the protein concentration is determined by use of the Bradford Reagent (Sigma-Aldrich) and a standard protein curve, following the Sigma 96 Well Assay Protocol as described in the Sigma Product Information sheet. This gives information about the sample concentration, and allows dilution of strong samples in order to give an even concentration of samples.

4.5 Gel Electrophoresis

20 µl of each of the samples were loaded into the wells of a 1mm thick Novex Tris-glycine 8-16% gradient gel. A molecular mass marker (Wide Range SigmaMarker™) was run on each gel. Electrophoresis was performed in a BDH VGT3 gel tank (BDH Merck) using a Consort power pack for 50V for 1 hour and then 150V for a further 2 hours. The gels were stained for total protein using Coomassie Blue, photographed and analyzed.

5. DISCUSSION

One of the main problems encountered has been with inequalities in proteins concentrations leading to problems analyzing gels. This can be seen in Figure 1, where two lanes from a gel are shown, illustrating the effect of protease inhibitor on the spontaneous fragmentation of the protein. It can be seen that despite the samples being prepared in the same fashion at the same time, there is a marked difference in the intensity of the bands between the two samples.

![Figure 1](image)

Figure 1: An example of the effects of digestion on collagen. Lane 2 shows collagen digested with 1 mg/ml collagenase, lane three shows the effect when the collagenase is neutralised by a protease inhibitor cocktail.

Lane 2 is partially saturated, whilst lane three is very faint, with some of the less distinct bands almost impossible to visualize. It is hoped that equilibration of the protein concentrations before they are loaded onto the gel will solve this problem.

Figure 1 is a very good example of why the protease inhibitor is needed, as it can be seen that the bands around 23 kDa have been digested further in lane 2 where protease inhibitor has been present, with strong bands around 20 kDa and even some fragments present down to around 7 kDa. Clearly this shows how additional digestion could completely
change the appearance of the protein fragments, and how enzymic digestion could completely invalidate any ablation results unless inhibited.

5.1 Ablation results

The necessity for protein equilibration to allow for effective analysis of ablation results becomes even more apparent when actual laser ablation results are examined. Figure 2 shows results from previous work at FELIX. It can clearly be seen that there is considerable saturation of some bands, whilst others are so under saturated as to make it extremely difficult to see anything except the main bands.

Clearly a lot of information has been lost on this gel due to the concentration problems. It is possible, with image manipulation, to show that there is a difference in the band structure between the 6.2 mm radiation in lane 4 and the 6.45 mm in lane 8, but it is not possible to pick out any fine detail from the lanes due to the over saturation. In addition, the lanes that were overloaded spilled into adjacent lanes, forcing the entire gel to run in a fan formation and resulting in early termination of the gel run and reducing the separation of the bands. Lanes loaded with an appropriate concentration of protein would avoid this effect and allow for accurate analysis of the gel.

Another potential benefit of this technique would be to allow for more accurate evaluation of the different concentrations within a molecular weight structure. For example, it can be seen in Figure 3 that while bands at around 22 kDa are over saturated, the bands of interest at around 97 kDa are under saturated to the point of being impossible to read.

Previous techniques have limited what can be done about this, as the extraction technique yields approximately 50 µl of sample buffer containing protein per sample. 20 µl of this is needed to load the gel, so in theory it is possible to load two gels with the sample available. This could be utilized in situations such as below in Figure 3 to allow for two gels to be run, one to allow for the heavy bands to be analyzed and one for the lighter bands where the samples have been concentrated.

It was hoped to have examples of gels prepared using the new technique prepared in time for inclusion with this paper, but this has unfortunately not been possible. Gels prepared with the new technique will hopefully be presented as a late poster at Photonics West 2002.
Figure 3. Gel showing over saturation of some bands whilst others remain sufficiently under saturated as to be hard to evaluate. In particular, the bands highlighted by the arrow at 97 kDa are of interest but cannot be clearly seen.

6. CONCLUSION

Electrophoresis is a potentially valuable tool for the analysis of ablation products, as it allows a direct analysis of the molecular makeup of ablation products. The molecular effects of the laser energy can be studied, even potentially at sub-ablation threshold energies. This will allow some interesting analysis of several issues that have been the topic of interest for some time.

Uhlhorn and others have postulated that the superior cutting performance shown at 6.45 µm is mainly a function of the unique pulse structure of the Free Electron Laser, and not a wavelength effect. By use of a conventional source and an FEL at the same wavelength the molecular effects both below and above the ablation threshold can be studied and compared, and any differences noted and investigated.

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8. REFERENCES
